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Titre de la thèse:

Assessment and relevance of the putative DNA/RNA helicase Schlafen-11 in ovarian and breast cancer

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Titre en Français

Évaluation et pertinence d'une putative hélicase ADN / ARN Schlafen-11 dans le cancer de l'ovaire et du sein.

Résumé en Français

Schlafen 11 (SLFN11) est une putative hélicase ADN / ARN, décrite pour la première fois pour son rôle dans le développement et la différenciation des thymocytes dans des modèles murins. La protéine SLFN11 fait partie d'une famille de protéines présentant divers degrés d'homologie selon les espèces, mais constamment présente chez les vertébrés et en particulier chez les mammifères. Récemment, le rôle de cette putative hélicase ADN / ARN, SLFN11, a été causalement associe à la sensibilité aux agents endommageant l'ADN, tels que les sels de platine, les inhibiteurs de la topoisomérase I et II et d'autres alkylateurs testes dans le panel de lignées de cellules cancéreuses NCI-60.

Dans notre première étude, nous avons validé un anticorps anti-SLFN11 dans des échantillons de carcinome séreux de l'ovaire de haut grade (HGSOC) inclus dans du formol (FFPE), en développant un protocole d'immunohistochimie (IHC) afin de déterminer l'expression de SLFN11 dans notre série de HGSOC.

En effet, nous avons testé et validé, en IHC, dans un bloc de cellules en culture (CCB) du carcinome de l'ovaire et dans une série indépendante de HGSOC en tissue micro-array, un fiable anticorps (Ab) anti-SLFN11 choisissant entre deux anticorps anti-SLFN11 utilisés normalement pour Western Blot (WB).

Pour chaque cas, nous avons évalué le score d'intensité (IS) et le score de distribution (DS) évaluant au moins 300 cellules. Un score histologique (HS) a été obtenu comme suit: HS = IS x DS.

Nous avons successivement appliqué notre protocole à une nouvelle et plus large série d'échantillons HGSOC afin de confirmer nos résultats préliminaires.

Nous avons trouvé un anticorps fiable dans les séries CCB et TMA, permettant de déterminer clairement l'expression de SLFN11 en IHC. Ces résultats ont été confirmés dans notre suivante et plus large série d'échantillons de FFPE HGSOC.

En résumé, en ce qui concerne les séries indépendantes TMA, nous avons constaté que la HS pour l'expression de SLFN11 présente une distribution normale bien qu' une expression intermédiaire est prévalente (≈ 60%). En manière spéculaire, SLFN11 n'a pas été exprimée dans pratiquement 40% des cas, ce qui correspond cliniquement aux patients résistants au platine dans environ 60% des cas (16/27).

Nous pensons donc que la faible expression IHC de SLFN 11 pourrait être corrélée à la réponse à la chimiothérapie à base de platine.

Nous estimons que nos travaux soutiennent qu'un protocole en IHC clair et spécifique permettant de déterminer l'expression de SLFN11 dans FFPE HGSOC par un anti-SLFN11 modifié, car normalement utilise en WB, a été mis en place.

Dans notre deuxième étude, nous avons étudié le panorama transcriptionnel de la protéine SLFN11 dans le cancer du sein en réalisant une méta-analyse par micro-array de l'expression génique de plus de 7 000 cas dérivant de 35 données publiquement disponibles.

Par l'analyse de corrélation, nous avons identifié 537 transcrits qui présentaient le 95e centile de coefficients de Pearson avec SLFN11 et qui identifiant la « réponse immunitaire», «l'activation des lymphocytes» et «l'activation des lymphocytes T» parmi les principaux processus defini par Gene Ontology. En outre, nous avons signalé une très forte association de la SLFN11 avec les signatures immunitaires dans le cancer du sein voie un penalized maximum like-lihood lasso regression.

Enfin, grâce à multiple corresponde analysis, nous avons découvert un sous-groupe de patients, défini comme "*SLF11-hot cluster"*, caractérisé par des taux élevés de SLFN11, une négativité des récepteurs des œstrogènes (RE), basal-like phenotype, une CD3D élevée, une signature STAT1 et un jeune âge, mais, en utilisant a Cox proportional hazard regression, nous avons individué les niveaux élevés de SLFN 11, l'indice de prolifération élevé et la négativité RE en tant que paramètres indépendants pour un intervalle plus long sans maladie chez les patients soumise a' chimiothérapie.

Nous estimons que nos travaux soutiennent la validation des suivants hypotheses : i) un rôle clair et spécifique du gène SLFN11 dans le cancer du sein, en relation probable avec la modulation du système

immunitaire dans cette maladie, ii) une forte corrélation entre un SFLN élevé et un sous-type moléculaire spécifique de cancer du sein (négativité RE, Basal-like phénotype).

D'autres études précliniques et cliniques doivent être menées pour confirmer notre hypothèse dans le cancer de l'ovaire et du sein.

Title in English:

Assessment and relevance of the putative DNA/RNA helicase Schlafen-11 in ovarian and breast cancer

Abstract in English

Schlafen 11 (SLFN11) is a putative DNA/RNA helicase, first described for its role in thymocyte development and differentiation in mouse models [1]. SLFN11 is part of a family of proteins with various degree of homology across species, but intriguingly being consistently present only in vertebrates and especially in mammals. Recently the role of this putative DNA/RNA helicase, SLFN11, was causal association with sensitivity to DNA damaging agents, such as platinum salts, topoisomerase I and II inhibitors, and other alkylators in the NCI-60 panel of cancer cell lines.[13]

In the first study, we validate an anti-SLFN11 antibody in formalin-fixed paraffin-embedded (FFPE) high-grade serous ovarian carcinoma (HGSOC) samples, developing a immunohistochemistry (IHC) protocol in order to determinate the expression of SLFN11 in our series of HGSOC.

Indeed, we tested and validated a reliable SLFN 11 antibody (Ab) in IHC choosing between two anti-SLFN11 Ab used normally for Western Blot (WB) in culture cell block (CCB) of ovarian carcinoma and in an independent series of HGSOCs tissue micro-array (TMA).

For each case, we evaluated both the Intensity Score (IS) and the Distribution Score (DS) evaluating at least 300 cells. A Histological Score (HS) was obtained as follow: HS=IS x DS.

Successively, we applied our protocol to a large case series of HGSOC samples to confirm our preliminary results.

We found one antibody to be reliable in CCB and TMA series allowing to determinate clearly IHC expression of SLFN11. These results were confirmed in our large case series of FFPE HGSOC samples.

Briefly, as for TMA independent series, we found that the HS for SLFN11 expression presents a normal distribution with a prevalent (≈ 60%) intermediate expression. Parallel SLFN11 was not expressed in practically 40% of cases that clinically corresponded to the platinum resistant patients in about 60% of cases (16/27).

So, we believe that low IHC expression of SLFN 11 should be correlated to response to the platinum based chemotherapy.

In the second study, we investigate the transcriptional landscape of SLFN11 in breast cancer performing a gene expression microarray meta-analysis of more than 7000 cases from 35 publicly available data sets.

By correlation analysis, we identified 537 transcripts in the top 95th percentile of Pearson's coef- ficients with SLFN11 identifying *"immune response", "lymphocyte activation"*and *"T cell activation"* as top Gene Ontology enriched processes. Furthermore, we reported very strong association of SLFN11 with immune signatures in breast cancer through penalized maximum like-lihood lasso regression

Finally, through multiple corresponde analysis we discovered a subgroup of patients, defined "*SLF11-hot cluster",* characterized by high SLFN11 levels, estrogen receptor negativity, basal-like phenotype, elevated CD3D, STAT1 signature, and young age and using Cox proportional hazard regression, we characterized SLFN11 high levels, high proliferation index, and ER negativity as independent parameters for longer disease-free interval in patients undergoing chemotherapy.

We believe that our work supports proof of concept that: i) A clear and specific role for SLFN11 in breast cancer, in likely connection with the immune system modulation in such disease entity, ii) a strong correlation between high SFLN 11 and specific molecular subtype of breast cancer (estrogen receptor negativity, basal-like phenotype).

Title:

Assessment and relevance of the putative DNA/RNA helicase Schlafen-11 in ovarian and breast cancer

1) INTRODUCTION

i) Schlafen Gene Family

Schlafen 11 is a putative DNA/RNA helicase belongs to the *Schlafen* gene family. These genes are highly conserved in murine and human species and they first identified in 1998 by a research group from the University of California led by Stephen Hedrick. These genes were named "*Schlafen*"(SLFN) from German, which means "to sleep", and it refers to the capacity to cause G0/G1 cell cycle arrest and to induce grown inhibition. [1] Currently, mouse *SLFN* genes consist of 10 members, SLFN1, SLFN1L, SLFN2, SLFN3, SLFN4, SLFN5, SLFN8, SLFN9, SLFN10, and SLFN14[2] and their genomic cluster maps on chromosome 11, while human *SLFN* genes, including SLFN5, SLFN11, SLFN12, SLFN12L, SLFN13, SLFN14, maps on chromosome 17. [3,4] Depending on their molecular weight and domain arrangement, SLFN proteins can be divided into three groups. (Figure N.1)

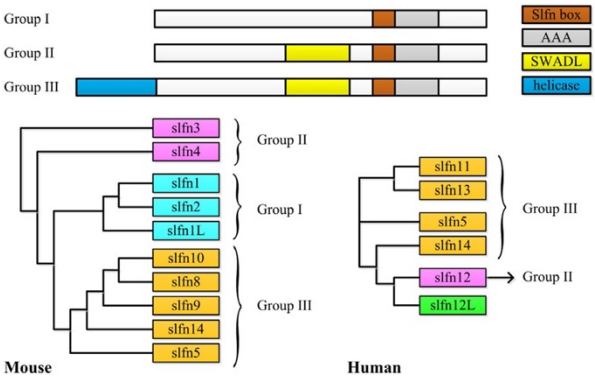


Figure N.1 Linear structural model of Schlafen family proteins.

The members of Schlafen family can be divided into three groups: group I ( ) includes m-slfn1, m-slfn1L, and m-slfn2; group II ( ) includes m-slfn3, m-slfn4, and h-slfn12; group III ( ) includes m-slfn5, m-slfn8, m-slfn9, m-slfn10, m-slfn14, h-slfn5, h-slfn11, h-slfn13, and h-slfn14. AAA structure and slfn box are common to all SLFN genes. SWADL belongs to groups II and III. Only group III contains helicase structure of C-terminal. Others ( ): h-slfn12 did not belong to the three groups.

Particular, Group I includes SLFN "short" proteins whose molecular weight range between 37 kDa and 42 kDa and it consists of SLFN1, SLFN1L, SLFN2. Group II includes SLFN "intermediate" proteins with molecular mass ranging from 58 kDa to 68 kDa and it comprises SLFN3 and SLFN4.

While in the Group III are found most of the SLFN "long" proteins, sizing between 100 kDa and 104 kDa. The members of this group are the only SLFN proteins characterized by a C-terminal domain homologous to the DNA/RNA helicases of superfamily I. [5-7] In addition, the C-terminal domain contains a nuclear localization motif RKRRR, suggesting a nuclear function for these proteins. [8] All the SLFN proteins share an N-terminal AAA domain, with presumable ATP/GTPase function [2]and a specific domain called "Slfn-box", adjacent to that mentioned above, whose function is unclear. [9,10] The intermediate and long SLFN proteins also share a highly conserved SWADL motif, consisting of a unique five-amino-acid sequence Ser-Trp-Ala-Asp-Leu. Unlike the murine SLFN proteins, that are distributed through the three groups, the human SLFN proteins belong to the Group III, with the exception of SLFN12 and SLFN12L included in the Group II. [9,10] SLFN proteins play an important role in immune processes, cell growth and differentiation indeed they are preferentially expressed in lymphoid tissue and SLFN genes are involved in T cells maturation and differentiation [1]. Moreover, SLNF genes are inducible by type I interferons [11,12], Toll-Like Receptor ligands [13] and it has been demonstrated to hamper viral replication. [12] Finally, a recent review summarizes the different functions of SLFN family in five different aspects: 1) Regulating cell proliferation, 2) Modulating the differentiation of T cells and macrophages, 3) Inhibition of invasion of cancer cells and sensitization cancers to chemotherapy (CT), 4) Inhibition of viral replication, 5) Participating in inflammatory response induced by Type I Interferon signal and mediated mitogen-activated protein kinase (MAPK)-integrating kinase (Mnk) and MAPK pathway.

Furthermore, the localization of each SLFN protein possesses functional specificity [9]. In mouse, the cytosolic proteins (SLFN1, SLFN2, SLFN3, and SLFN4) modulate growth arrest and the nuclear proteins active the RNA polymerase II[8]. However, in humans, the relative functions and the specific location of SLFN proteins must be clearly defined. Anyway, SLFN5 has been localized in the nucleus [11], while SLFN12 and SLFN11 seem be localized in the cytosol. [12,14] Although SLFN

proteins involvement in cell growth process is not yet fully understood, it was reported that SLFN11 exerts growth inhibitory effects by blocking cyclin D1 promoter activity.[15]

To date, most of the research has been carried out on murine SLFNs. Among human SLFNs members, SLFN11 has been studied more extensively and it is getting more and more significant, mainly in the oncological field. Interestingly, lack of expression SLFN 11 has causally been associated with resistance to several types of DNA damaging agents (DDA), including topoisomerase (TOP) I (topotecan and irinotecan) and II (doxorubicin, mitoxantrone, etoposide) inhibitors, alkylating agents (chlorambucil, cisplatin), DNA synthesis inhibitors (gemcitabine and fludarabine) and poly(ADP-ribose) polymerase(PARP) inhibitors. [16-22]

ii) DNA-TARGETING ANTICANCER DRUGS

To date, DNA-damaging chemotherapeutic agents constitute the backbone of treatment for most solid and hematological tumors but no individual biomarker has been shown to be superior to tumor clinical stage and pathological features in predicting treatment response.

a) Platinum-Compounds

Cisplatin and its analogs (carboplatin and oxaliplatin) have been defined the milestone for treatment numerous human cancers including bladder, head and neck, lung, ovarian, and testicular cancers. They are effective against various types of cancers, including carcinomas, germ cell tumors, lymphomas, and sarcomas. Their mode of action have been linked to their ability to crosslink with the purine bases on the DNA; interfering with DNA repair mechanisms, causing DNA damage, and subsequently inducing apoptosis in cancer cells. [23]

1) Cisplatin

Cisplatin is one of the most potent chemotherapy drugs widely used for cancer treatment. The discovery of cisplatin, cis-[Pt(II)(NH(3))(2)Cl(2)] ([PtCl2(NH3)2] or CDDP), was a cornerstone which triggered the interest in platinum and other metal-containing compounds as potential anticancer drugs. Cisplatin covalently binds to DNA and disrupts DNA function. After cisplatin enters the cells, the chloride ligands are replaced by water molecules. This reaction results in the formation of positively charged platinum complexes that react with the nucleophilic sites on DNA. These platinum complexes covalently bind to DNA bases using intra-strand and inter-strand cross-links creating cisplatin-DNA adducts thus preventing DNA, RNA and protein synthesis. This action is cell cycle phase-nonspecific. [23]

2) Carboplatin

Carboplatin or Cis diammine (1,1-cyclobutanecarboxylato) platinum is a chemotherapeutic drug used for cancers of ovaries, lung, head, and neck. In terms of its structure, carboplatin differs from cisplatin in that it has a bidentate dicarboxylate (CBDCA) ligand in place of the two chloride ligands, which are the leaving groups in cisplatin (Figures 1 and 2). It exhibits lower reactivity and slower DNA binding kinetics, although it forms the same reaction products in vitro at equivalent doses with cisplatin. The lower excretion rate of carboplatin means that more is retained in the body, and hence its effects are longer lasting (a retention half-life of 30 hours for carboplatin, compared to 1.5-3.6 hours in the case of cisplatin). Relative to cisplatin, the greatest benefit of carboplatin is its reduced side effects, particularly the elimination of nephrotoxic effects. The main drawback of carboplatin is its myelosuppressive effect which causes the blood cell and platelet output of bone marrow in the body to decrease quite dramatically, sometimes as low as 10% of its usual production levels. Carboplatin is less potent than cisplatin; depending on the type of cancer, carboplatin may only be 1/8 to 1/45 as effective. The clinical standard of dosage of carboplatin is

usually a 4:1 ratio compared to cisplatin; that is, for a dose that usually requires a particular dose of cisplatin, four times more carboplatin is needed to achieve the same effectiveness.

b) Topoisomerase Inhibitors

TOP inhibitors are agents designed to interfere with the action of topoisomerase enzymes (TOPI and II), which are enzymes that control the changes in DNA structure by catalyzing the breaking and rejoining of the phosphodiester backbone of DNA strands during the normal cell cycle. DNA topoisomerases are ubiquitous enzymes that catalyze essential enzymes to solve the topological problems accompanying key nuclear processes such as DNA replication, transcription, repair, and chromatin assembly by introducing temporary single or double strand breaks in the DNA. TOP inhibitors include catalytic inhibitors of the enzyme and topoisomerase poisons. TOP poisons exert their cytotoxic effects by stabilizing the covalent complexes between enzyme and DNA (cleavable complex). These compounds interfere in the religation step of the enzyme catalysis, thereby, leaving the DNA strand breaks unligated. The protein-DNA strand breaks thus created are not efficiently repaired and induce apoptosis. On the other hand, the catalytic inhibitors inhibit the enzyme catalysis activity by not allowing the enzyme to function itself and therefore do not allow topoisomerases to create strand break. Several compounds, membrone, suramin, bisdioxopiperazines (ICRF), are known catalytic inhibitors of topoisomerase, while epipodophyllotoxins like etoposide (topo-II inhibitor) and camptothecin (topo-I inhibitor) are well-known topoisomerase poisons.

TOPI inhibitors include irinotecan, topotecan, and camptothecin, and TOP II inhibitors include etoposide, doxorubicin, and epirubicin. [24]

1) Type I topoisomerase inhibitors

The TOPI inhibitors topotecan and irinotecan are commonly used alone or combination with platinum therapy during the first or second line of treatment in several cancers. The Top I enzyme class is responsible for creating single strand breaks in DNA to relieve torsional strain created by

twisting and supercoiling. Binding of topotecan or irinotecan to the Top I-DNA complex prevents repair of these single-strand nicks and, ultimately, to unrepaired double-strand breaks and apoptosis.[24]

2) Type II topoisomerase inhibitors

Etoposide is also widely used in combination with platinum-chemotherapy during the first or second line of treatment in several cancers. Etoposide targets Top II, an enzyme class that plays a critical role during DNA replication. Specifically, Top II cleaves double-stranded DNA to permit passage of intact helical DNA before ligating the cleavage site. Etoposide prevents this ligation event by stabilizing the complex formed by Top II and the 5' cleaved ends of the DNA, resulting in stable, protein-linked double-strand breaks in DNA and subsequent apoptosis. [24]

c) Parp Inhibitors

Poly (ADP-ribose) polymerase (PARP) inhibitors are nuclear enzymes, whose main role is to detect and mark single-strand DNA breaks (SSB) by signaling the enzymatic machinery involved in the SSB repair. They block PARP enzyme activity, which catalyzes the attachment of adenosine diphosphate (ADP)-ribose to itself or its target proteins to form a poly ADP-ribose chain with nicotinamide adenine dinucleotide (NAD) serving as a donor. Poly ADP-ribosylation (PARylation) is one of the major protein modifications that occur in response to DNA damage. The binding of PARP1 to damaged DNA recruits DNA-repair-related proteins, such as XPA, XRCC-1, polymerase ε, MSH6 and DNA ligase III, to the DNA-damage sites through interaction with PARylated PARP1 to activate DNA repair. [25,26] Figure N.2

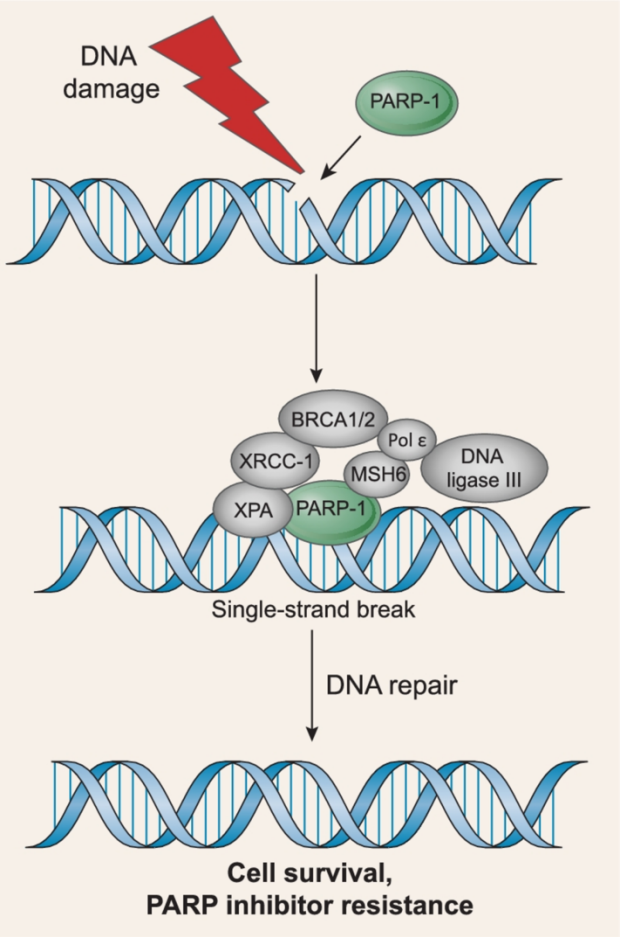


Figure N.2 Schematic of PARP1 in response to DNA damage.

There are at least two distinct mechanisms of action of PARP inhibitors: enzymatic inhibition and PARP trapping.[27]

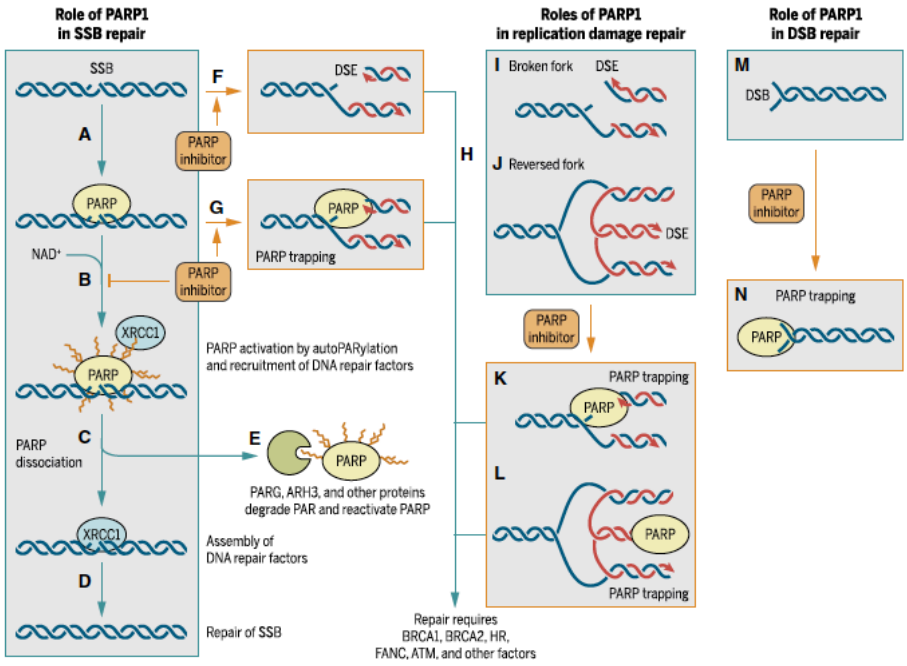


Figure N. 3 DNA repair by PARP1 and the effects of PARP inhibitors.

Upon the generation of an SSB, PARP1 binds to the break (A) and uses NAD^+ (B) to generate PAR polymers on itself (auto-PARylation), as well as on histones and chromatin-associated proteins. This serves the purpose of relaxing chromatin and recruiting repair proteins. Cumulative auto-PARylation causes the dissociation of PARP1 from DNA (C), allowing access to other repair factors scaffolded by XRCC1 (D). PARylation is removed by PARG (E), a glycohydrolase, which allows PARP1 reactivation. PARP inhibitors block NAD^+ binding and PARylation for as long as the inhibitor is bound to the NAD^+ site (B), thereby preventing PARP dissociation from the SSB, resulting in both accumulation of unrepaired SSBs (F) and PARP trapping (G). Repairing the ensuing DSB and PARP trapping will require BRCA1, BRCA2, and other HRR factors, as well as ATM, Fanconi, and replication bypass pathways for cell survival (H). PARP1 is also involved in the repair of "collapsed forks" with DSEs (I), in the retraction and restart of stalled replication forks (J), and in the repair of DSBs (M). PARP inhibitors trap PARP at DSEs (K and L) and DSBs (N).

Inhibition of PARP enzymatic activity was initially thought to explain the synthetic lethality observed with PARP inhibitors in breast and ovarian cancers with BRCA1, DNA repair associated (BRCA1) and BRCA2, DNA repair associated (BRCA2) mutations. These mutations lead to deficiencies in Homologous Recombination (HR), leaving these cancers highly dependent on PARP-mediated repair. [28,29]

The PARP inhibitor olaparib was recently approved for treatment of germline BRCA1/2 mutant ovarian cancer patients based on the results of a randomized phase II study. [30]

PARP trapping is a distinct mechanism of action of PARP inhibitors, whereby the inhibitor/PARP complex becomes fixed on the DNA at sites of SSBs, leading to a failure to repair, and, with replication, induction of multiple double-strand breaks. PARP trapping may be

responsible for synergy between PARP inhibitors and DDA that increase the prevalence of SSBs. (28) Further, this mechanism may be operant in cancers without defined HR deficiencies. [27]The various PARP inhibitors in clinical development and clinical use vary in relative potency for both enzymatic inhibition and PARP trapping effects. Olaparib and talazoparib have comparable levels of catalytic inhibition, while talazoparib is 100-fold more potent than olaparib at trapping PARP–DNA complexes. Rucaparib appears to have activity similar to olaparib, while veliparib is less potent both in enzymatic inhibition and in trapping activity. [27,31]

Beyond inactivating mutations in known mediators of HR such as BRCA1/2, other mechanisms may result in HR deficiency in sporadic tumors, including epigenetic silencing of BRCA1 and HR pathway disruptions in other known and unknown mediators of this pathway. [32,33]

This has led to substantial interest in strategies for defining "BRCAness," or HR deficiency (HRD), including using characteristic patterns of mutation and loss from whole-exome sequencing data to generate HRD scores. [34-36]

Today, BRCA mutations are currently the only biomarkers of response to PARP inhibitors used in the clinic setting but several studies are investigating to discover new potential biomarker because the response to PARP inhibitors did not always correlate with the BRCA status. [37,38] Indeed, in pre-clinical studies, HR repair proteins (p53, ATM and 53BP1) have been shown to predict PARP inhibitor response in breast, lung and gastric cancers and the loss of SLFN 11, which encodes a putative DNA/RNA helicase, was shown to confer resistance to talazoparib in Ewing's sarcoma and small-cell lung cancer. [21,39-41]

2) SCHLAFEN-11

i) General description, discovery, and development

In the year 2012, two authors, working in the domain of oncologic research, discovered independently a new protein, Schlafen11(SLFN11), that showed a putative DNA/RNA helicase function, whose expression was correlated to the sensitivity of response to DDA. [16,17] The gene of SLFN 11 is located on chromosome 17q12. [3,4]

Precisely, Barretina et al., in order to individuate predictive markers of response to several chemotherapies agents, developed the Cancer Cell Line Encyclopedia (CCLE) that correlates a large-scale genomic dataset from 947 human cancer cell lines with pharmacological profiles of 24 anticancer drugs. It represents much of the diversity of human cancers including data from both common and rare cancer types. Furthermore, each cell line was genetically characterized through a series of high-throughput analyses, including whole genome, whole exome, and RNA sequencing. Interestingly, CCLE presents a strong positive correlation in chromosomal copy number, gene expression and mutation frequencies with primary tumor derived from Tumorscape, Mile, exp0 and Cosmic allowing to create representative genetic proxies for the primary tumor in many cancer types. Finally, by analyzing the CCLE, they identified that SLFN 11 expression represented the top predictor of response to topoisomerase I agents (irinotecan and topotecan) and then, they validated these finding from the NCI-60 collection[16] that encompasses 60 human cancer cell lines from nine different tissues of origin: breast, colon, skin, blood, central nervous system, lung, prostate, ovary, and kidney. It has been tested since the 1980s for more than 400,000 compounds of natural and synthetic origin. [42-44] Moreover, the NCI-60 panel has also been extensively characterized for gene expression using six different microarray platforms [45-47]and copy-number variation by array-based comparative genomic hybridization (aCGH) [43,48] and has recently been sequenced for the entire exome at the National Cancer Institute (National Institutes of Health). Fig N.4

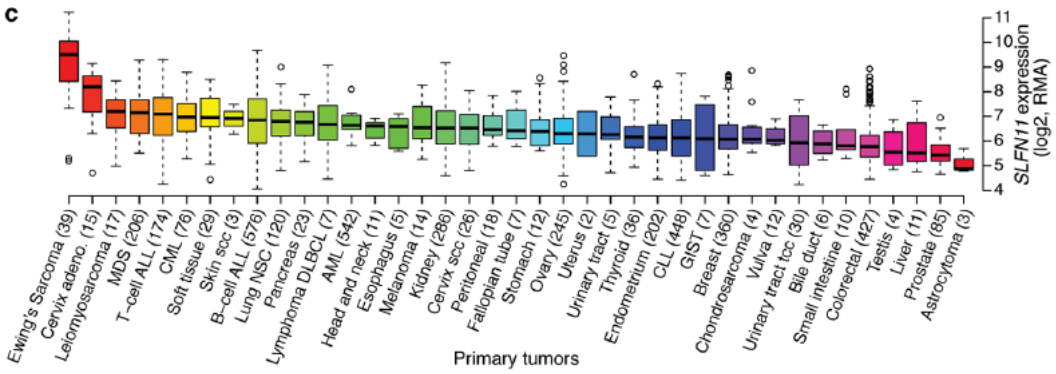


Figure N.4 ***SLFN11 expression across 4103 primary tumors.***

Box-and-whisker plots show the distribution of mRNA expression for each subtype, ordered by the median *SLFN11* expression level (line), the inter-quartile range (box) and up to 1.5x the inter-quartile range (bars). Sample numbers (n) are indicated in parentheses.

At the same time, these data were independently confirmed by Zoppoli et al. that, correlating the transcriptome of the NCI-60 Panel cancer cell lines with the cytotoxicity profiles of four Top1 inhibitors, identified one gene, SLFN11, which was highly correlated with their in vitro antiproliferative activity. Then, they amplified our analysis to 1,444 compounds in the NCI-60 and they observed significant positive correlations of SLFN11 expression with the cytotoxicity profiles of several FDA-approved DDA, including Top1 inhibitors (topotecan and irinotecan), Top2 inhibitors (doxorubicin, mitoxantrone, etoposide), DNA alkylating agents (chlorambucil, melphalan, cisplatin), and DNA synthesis inhibitors (gemcitabine and fludarabine), but not with drugs targeting other components of the cancer cell, such as protein kinases (erlotinib, sorafenib, dasatinib), tubulin (docetaxel, paclitaxel, vincristine), protein synthesis (L-asparaginase), or the proteasome (bortezomib).In summary, they showed that SLFN11 exerted a broader role in determining sensitivity to several DDA. [17]

In addition to its role in sensitizing malignant cells to TOP inhibitors, as well as alkylating agents and other DDA, SLFN 11 has been shown to have also important antiviral properties. In fact, in 2012, Li et al. discovered that SLFN 11 was interferon (IFN)- induced antiviral protein which acted as an inhibitor of retrovirus protein synthesis. It seems specifically abrogate the production of a retrovirus such as human immunodeficiency virus 1(HIV-1) by inhibiting, at the late stage, the

expression of viral proteins in a codon-usage-dependent manner[12]. The inhibitory activity resided in the 579-residue N-terminal half of SLFN11, which includes the AAA domain. SLFN11 does not inhibit reverse transcription, integration or production and nuclear export of viral RNA, nor block in budding or release of viral particles but it binds transfer RNA or counteracts changes in the tRNA poll elicited by the presence of HIV-1. In summary, the exact inhibition mechanism of SLFN 11 in HIV-1 is unclear. It may either sequester tRNAs, prevent their maturation via post-transcriptional processing or may accelerate their diacylation. Finally, SLFN11 remains potent and IFN-inducible restriction factor against retroviruses that mediates its antiviral effects on the basis of codon usage discrimination. [12]

Thus, in 2012, SLFN 11 turned out as an interesting topic in the research world and, in particular, in the oncological domain, without that his function had not yet been understood.

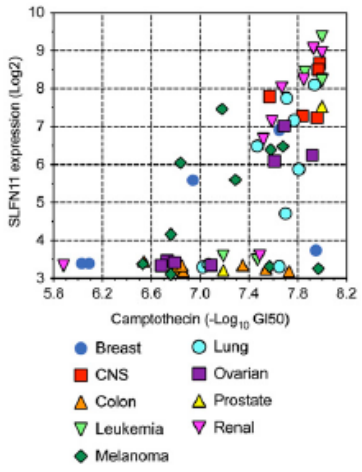
Thus, in the following years, different studies have been performed in order to understand the real value of SLFN 11 as a predictive marker of chemo-response and his functional mechanism in oncology.

ii) In Vitro Studies

The value of SLFN 11 as predictor to response to the DDA has been recently assessed in various preclinical studies that are described below.

Barretina et al., in their CCLE, described as Ewing's sarcoma cell lines had very high SLFN11 mRNA expression and, that a tight correlation existed between SLFN 11 transcript expression and TOP 1 inhibitor toxicity in cancer cells. [16] Zoppoli et al. determined as the expression of a single gene, SLFN11, showed an extremely significant positive correlation with the response DDA. Figure N. 5B In the same paper, they measured, by Western Blot (WB), SLFN11 expression in several NCI-60 cell lines assessing the relationship between SLFN11 transcript and SLFN11 protein levels

and confirming that the cell lines overexpressing SLFN11 transcript, such as DU-145 (prostatic cancer) and HOP-62 (non-small cell lung cancer) presented also high SLFN11 protein levels. Then, they measured SLFN 11 expression in lung, colon, breast, and prostate cancer lines (HOP-62, HCT-116, MDA-MB-231, and DU-145 respectively), thereby demonstrating the causal relationship between SLFN11 intracellular levels and sensitivity to DDA. [17] **Figure N. 5A**



NSC*	Chemical name†	Mechanism of action	r‡	P value§	FDR¶
Compounds with significantly positive correlation with SLFN11 transcript					
609699	Topotecan	Topoisomerase I inhibitor	0.714	2.2×10^{-10}	1.9×10^{-9}
616348	Irinotecan	Topoisomerase I inhibitor	0.613	2.5×10^{-7}	7.3×10^{-6}
301739	Mitoxantrone	Topoisomerase II inhibitor	0.624	1.3×10^{-7}	4.5×10^{-6}
141540	Etoposide	Topoisomerase II inhibitor	0.533	1.4×10^{-5}	2.2×10^{-10}
82151	Daunorubicin	Topoisomerase II inhibitor	0.399	0.002	0.018
v123127	Doxorubicin	Topoisomerase II inhibitor	0.387	0.002	0.024
3088	Chlorambucil	Alkylating agents at N7 guanine	0.750	8.1×10^{-12}	1.2×10^{-9}
8806	Melphalan	Alkylating agents at N7 guanine	0.680	3.2×10^{-9}	3.3×10^{-9}
119875	Cisplatin	Alkylating agents at N7 guanine	0.619	1.7×10^{-7}	5.4×10^{-6}
241240	Carboplatin	Alkylating agents at N7 guanine	0.488	8.9×10^{-5}	0.001
409962	Carmustine	Alkylating agents at O6 guanine	0.439	0.001	0.006
613327	Gemcitabine	DNA synthesis inhibitor	0.669	7.0×10^{-9}	7.0×10^{-9}
V63878	Cytarabine	DNA synthesis inhibitor	0.644	3.8×10^{-8}	1.5×10^{-6}
Compounds with nonsignificant correlation with SLFN11 transcript					
67574	Vincristine	Tubulin active antimitotic agent	0.067	n.s.	n.s.
49842	Vinblastine	Tubulin active antimitotic agent	0.054	n.s.	n.s.
125973	Taxol	Tubulin active antimitotic agent	−0.100	n.s.	n.s.
718781	Erlotinib	Kinase inhibitor	0.147	n.s.	n.s.
732517	Dasatinib	Kinase inhibitor	0.041	n.s.	n.s.
750690	Sunitinib	Kinase inhibitor	0.001	n.s.	n.s.
747971	Sorafenib	Kinase inhibitor	−0.075	n.s.	n.s.
715055	Gefitinib	Kinase inhibitor	−0.090	n.s.	n.s.
745750	Lapatinib	Kinase inhibitor	−0.093	n.s.	n.s.
743414	Imatinib	Kinase inhibitor	−0.167	n.s.	n.s.
747599	Nilotinib	Kinase inhibitor	−0.212	n.s.	n.s.
733504	Everolimus	mTOR inhibitor	0.160	n.s.	n.s.
109229	L-asparaginase	Protein synthesis inhibitor	−0.086	n.s.	n.s.
681239	Bortezomib	Proteasome inhibitor	−0.123	n.s.	n.s.

A **B**

Figure N.5 ***SLFN11 expression is highly correlated with the in vitro antiproliferative activity of DDA***.

A) Scatterplot showing the correlation between SLFN11 expression (y axis, Log_2 intensity) and CPT antiproliferative activity (x axis, negative Log_{10} growth inhibitory molar concentration 50%, GI50) in the NCI-60; **B)** Correlation is between the in vitro activities of commonly used FDA-approved anticancer drugs and SLFN11 transcript across the NCI- 60. n.s., not significant.

Precisely, in order to confirm their hypothesis concerning the predictive value of chemo-response of SLFN 11, they tested the efficacy of several CT on cancer cell lines that normally show high expression levels of SLFN11, such as DU-145 and HOP-62. The same drugs were then tested on the same cancer cell lines (DU-145 and HOP-62) after SLFN11 siRNA-mediated silencing. These silenced cells showed at least a fivefold reduction in sensitivity to etoposide and cisplatin but not for tubulin poison paclitaxel or the broad-spectrum protein kinase inhibitor and apoptosis inducer staurosporine confirming the correlation between SLFN 11 and sensitivity to DDA. [17] FIGURA N.6

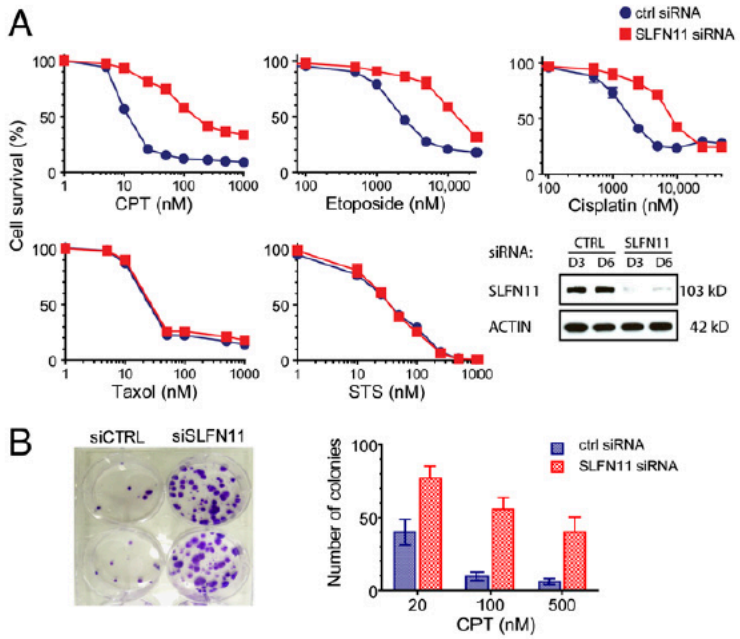


Figure N.6 ***Silencing SLFN11 significantly reduces sensitivity to different classes of DNA-damaging agents in cells expressing high endogenous SLFN11 levels.***

A) Cytotoxicity curves of the prostate cancer cell line DU-145 (high SLFN11 expresser) transfected with nontargeting (ctrl) or SLFN11-targeting siRNAs and treated for 72 h with CPT, etoposide, cisplatin, taxol, or staurosporine (STS). Mean values ± SD are shown (one representative experiment performed in triplicate). (Lower Right) Western blot showing SLFN11 knockdown 3 and 6 d after transfection with SLFN11-targeting siRNAs. **B)** (Left) Representative image of a clonogenic assay (100 nM CPT for 1 d). (Right) The number of colonies formed after 24 h treatment with CPT followed by a 15- d release (average of three independent experiments).

Wang et al., through their PC-Meta, a statistical framework based on the meta-analysis of expression profiles used to identify pan-cancer markers and mechanisms of drug response, showed that SLFN11 gene expression was increased in cell lines sensitive to both Topotecan and Irinotecan defining this last one as top marker of chemo-response to TOP I. [49]

Others in vitro studies confirmed the association between SLFN11 and DDA trying to understand its mechanism of action.

Tian et al. described the correlation between SLFN11expression and SN-38, a metabolite active of irinotecan, in colorectal cancer (CRC) cell lines. They detected SLFN11 mRNA and protein levels in eight human CRC cell lines. Precisely, SLFN11 mRNA and protein levels varied markedly between the several CRC cell lines (high expression of SLFN11 in LS174T and SW480 cells, whereas a lower SLFN11 expression level in the HCT-8 and HCT-116). Finally, they showed as

high expression of SLFN 11 was correlated to sensitivity to TOP I inhibitors because silencing two highSLFN11 expression cell lines (LS174T and SW480) they showed reduced sensitivity to SN-38 compared with control cells (down-regulation). In the other side, upregulation of SLFN11 expression in low SLFN 11 expression cells (HCT-8 and HCT-116) showed an increased sensitivity to SN-38 treatment. Furthermore, SLFN 11 seems to play a key role in cell cycle arrest and/or induction of apoptosis in response to exogenous SN-38-induced DDA because overexpression of SLFN11 in the two low-SLFN11expressing cell lines, HCT-8, and HCT-116, caused G0/G1 arrest in response to SN-38. [50]

Sousa et al., using the elastic net regression method, which reconstructs drug activity patterns through linear regression and integration of genomic features, found hundreds of potential interactions between genomic features of DNA genes and drug activity; the vast majority of these interactions involved DDA. A remarkable result was the strong association of SLFN11 expression and the activity of 147 out of 242 DDA (76 TopI inhibitors, 29 alkylating agents, 20 TOP II inhibitors, 17 DNA synthesis inhibitors, and 5 other DDA) whereas none of the 402 non-DDA showed significant association with SLFN11. [18]

This result continues to demonstrate the importance of SLFN11 for response to DDA as a probable predictive biomarker in different types of tumor and, in the last years, several researchers are performing interesting translational studies to clear the function of SLFN11 and in which types of tumor it could be used as a predictive biomarker.

iii) Translational Studies

1) Ewing's Sarcoma

After the above-mentioned paper of Barretina et al., several authors described the relationship between the sensitivity of irinotecan and SLFN11 expression in Ewing's

sarcoma (EWS) cell lines and tumor samples.

Tang et al. showed that SLFN11 expression is transcriptionally activated by following ETS transcription factors: EWS-FLI1 and ETS1. Precisely, the chimeric transcription factor EWS-FLI1 preferentially binds at the transcription start site of SLFN11, activates the SLFN11 promoter and regulates the expression of SLFN11 mRNA and their corresponding protein. Indeed, they showed that the EWS-FLI1–overexpressing HT1080 cells had selectively enhanced in the induction of SLFN11 promoter activity producing an increased expression of SLFN 11 mRNA and relative protein compared to control HT1080cells. They measured SLFN 11 mRNA and protein expression by quantitative real-time PCR (qRT-PCR) and by WB, respectively. In order to confirm this hypothesis, the authors showed a decrease of EWS-FLI1 and SLFN11 expression when EWS-FLI1–overexpressing HT1080 cells were silenced by sh-targeting EWS-FLI1. Furthermore, EWS patients presenting the higher SLFN11 expression, exhibited better prognosis than those with the lower SLFN11 expression inducing to consider SLFN11 as a probable prognostic marker of tumor-free survival in the EWS patients. Upregulation of SLFN11 expression by EWS-FLI1 enhances the sensitivity of EWS cells to camptothecin (transfection of A673 cells with SLFN11 siRNA increased resistance to camptothecin) and plays a role in the synergistic effects of PARP inhibitors with temozolomide (ASP14 cells with EWS-FLI1 knockdown and A673 cells with SLFN11 knockdown exhibited resistance to the combination of niraparib plus temozolomide). [41]

Kang et al. showed that, in a panel of cell lines and in primary tumors from Affymetrix Human Exon microarrays, SLFN11 mRNA expression, measured by qRT-PCR, was significantly higher for EWS relative to neuroblastoma or rhabdomyosarcomas. Furthermore, in 20 EWS cell lines, they observed a strong correlation between SLFN11 expression and SN-38 sensitivity. They confirmed their data in vivo study because they showed an improved response to nal-IRI (nanoliposomal

formulation of irinotecan, also known as MM-398 or PEP02) in EWS tumor xenograft presenting high SLFN 11 expression. [51]

Goss et al. showed that EWS cells, presenting a high expression of SLFN11, had increased the sensitivity to ribonucleotide reductase (RNR) inhibitors. This hypothesis is confirmed because the action of RNR is decreased in knockdown SLFN11 EWS cell lines. [52]

2) Ovarian Cancer

Platinum-based CT plays an important role in the treatment of ovarian cancer(OC). Predictive marker of chemo-response, that should allow stratifying the patients improving the management care, not yet available. So, SLFN 11 expression should be correlated with platinum sensitivity and different studies has been performed in order to demonstrate this association.

First, Zoppoli et al. described a probable correlation between SLFN 11 and response to platinum-based CT. They evaluated a publicly available well-annotated microarray dataset of 110 OC patients treated with a cisplatin-containing regimen after primary surgery (18). In univariate analysis, high SLFN11expressers patients had a median over-survival of 80 months compared to 49 months in low SFLN11 expressers patients. [17] Figure N. 7B

Then, Nogales et al., in DNA methylation analysis of NCI 60 cell line panel, identified that SLFN11 CpG promoter island hypermethylation was associated with an inactivation of SLFN11 gene expression in cancer cells inducing a decreased sensitivity to platinum (cis/carbo-platinum) based chemotherapy. In vitro, they determinate the following results:

1) SLFN11 presents a CpG island located around its transcription start site what makes it a candidate gene for hypermethylation-associated inactivation in human cancer; 2) The treatment of HCT15 cancer cell lines, that are hypermethylated at the SLFN11 CpG island and that had minimal expression of the SLFN11 RNA transcript, with a DNA-demethylating agent-induced an increase of SLFN11 mRNA and protein expression. 3) Downregulation of mRNA and protein SFLN11 expression in SK-OV3 cell lines, that normally had high SLFN 11 expression, induces a decrease

platinum sensitivity. Finally, these in vitro analyses were transferred into clinical samples demonstrating that, in a cohort of 41 cases of papillary serous OC, SLFN11 hypermethylation were significantly associated with shorter OS and PFS. [19] Figure A

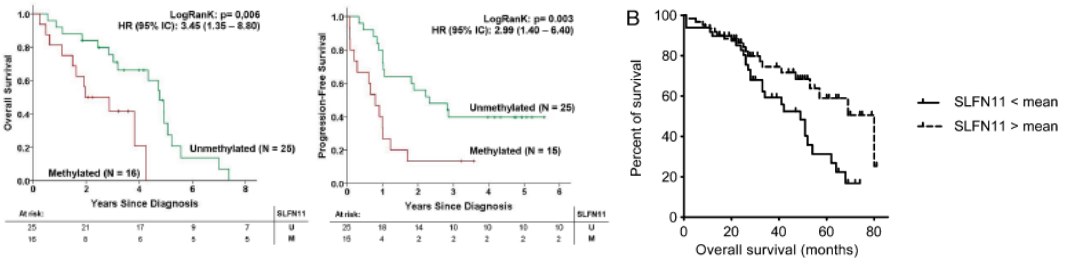


Figure N.7 A and B SLFN11 expression may predict overall survival and progression-free survival in ovarian cancer patients. A) Kaplan-Meier analysis of overall survival (OS) and progression-free survival (PFS) in the ovarian cancer clinical cohort with respect to SLFN11 methylation status. The statistical significance of the log-rank test is shown. Results of the univariate Cox regression analysis are represented by the hazard ratio (HR) and 95% confidence interval (95% CI). The number of cases (n) and the mean time to progression/survival in years (y) is indicated for each group; B) Kaplan–Meyer curves of 110 patients affected by ovarian cancer and treated with a first-line cisplatin-containing regimen. Patients are stratified as having higher or lower than average SLFN11 expression levels in that cohort (y-axis: percentage survival; x-axis: overall survival in months from diagnosis)

3) *Colorectal Cancer*

Irinotecan (CPT-11), a TOPI inhibitor, is one of the most important drugs in the treatment of advanced and/or metastatic CRC [2]. CRC presents a poor clinical response to conventional drugs and only 20–30% of patients show an objective response to CPT-11 [3]. Several studies showed that SLFN11 plays an important role in CRC cells because the sensitivity to TOP inhibitors is correlated with SLFN11 expression which should used as predictive biomarker of chemo-response in CRC patients. (53,54)

Indeed, He et al., in vitro, showed that SFN11 expression is strongly correlated with the sensitivity of CRC cells to cisplatin CT. The IC50 values of cisplatin vary in according to SLFN 11 expression and it was significantly increased after knockdown of SLFN11 in DKO cell line. Furthermore, the authors showed that, in vitro, SLFN11 suppressed CRC growth and proliferation because the amount of apoptosis induced by cisplatin treatment significantly increased after re-expression of SLFN11 and significantly decreased after knockdown of SLFN 11 in DKO cells. These data were

also confirmed in vivo in DLD1 cell xenograft mouse models where the volume and the height of the tumor decreased in SLFN 11 re-expressed respect to unexpressed control group. [53] Figure N.8

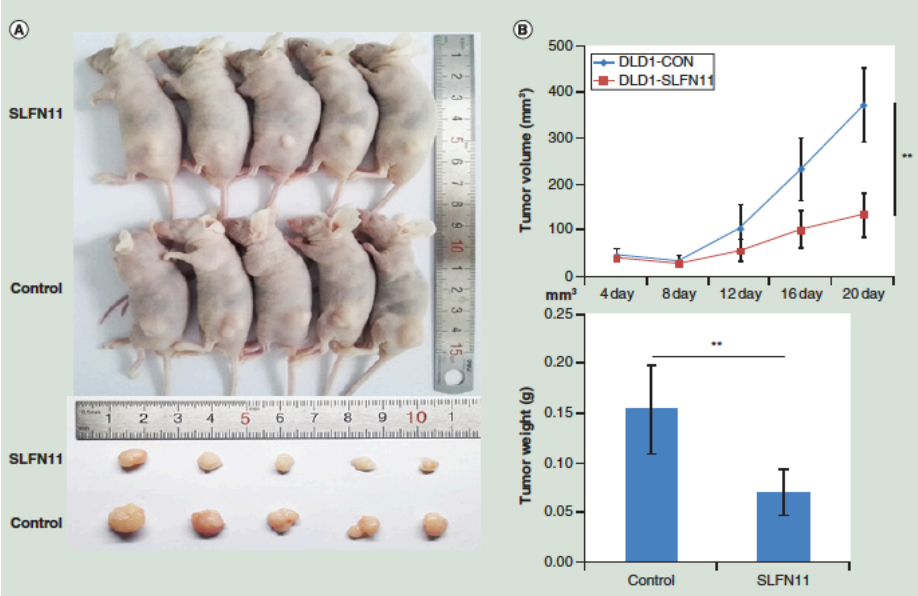


Figure N. 8 Inhibits tumor growth in colorectal cancer cell xenograft mice. (A)

Results of SLFN11 re-expressed and unexpressed DLD1 cell xenografts in mice – top: SLFN11 re-expressed colorectal cells group; Bottom: control group. **(B)** Tumor growth curves and average weights of SLFN11 re-expressed and unexpressed DLD1 cell xenografts. **p < 0.01.

Finally, they demonstrated, as Nogales e al., that the expression of SLFN11 is regulated by promoter region methylation that, if methylated, induced low SFLN11 expression. Furthermore, they showed that low SLFN11 expression was significantly associated with poor 5-year OS and 5-year RFS in CRC patients. So, SLFN11 methylation should be an independent prognostic factor for OS and RFS in CRC.

Interestingly, Deng et al. developed first immunohistochemistry (IHC) score to determinate SLFN 11 expression in CRC. The final score was calculated by intensity (scored as 0, none; 1, weak; 2: moderate; 3, strong) plus the proportion of positive tumor cells (<25 % positive= 0, 25–50 % positive=1, 50–75 % positive=2, 75–100 % positive=3). They studied SLFN11 expression in 271 stage II–III CRCs treated with oxaliplatin-based adjuvant CT (FOLFOX). Finally, they determined a cut-off point in a ROC curve in order to stratify their cohort of CRCs in Low vs High SLFN 11

expression CRC patients. Then, they found that high SLFN 11 expression was correlated with well/moderate CRC, Stage II CRC patients and tended to have better OS than those with low SLFN 11 expression. Interestingly, in a small cohort of stage II and III CRC patients presenting KRAS wild type, high SLFN11 expression have also better OS than those with low SLFN 11 expression considering, so, SLFN 11 as a possible predictive and prognostic marker of response to FOLFOX in this group of selected patients. [54]

4) *Lung Cancer*

Small cell lung cancer (SCLC) patients are initially highly responsive to cisplatin and etoposide but quickly develop a refractory disease. So, understanding the nature of CT resistance becomes necessary to develop new treatment and to discover new biomarkers.

First, Lok et al. investigated response predictors to PARP inhibitors and found that SLFN11 expression correlated with response. Indeed, by analyzing expression of 12,631 genes in 414 cell lines they identified that SLFN11 was among the top genes most significantly correlated with PARP inhibitors sensitivity and that SCLC cell lines with high levels of SLFN11 transcript were more sensitive to PARP inhibitors and to conventional cytotoxic therapy. Furthermore, loss of SLFN11, by silencing shRNA sequences targeting SLFN11, confered resistance to PARP inhibition in SCLC cell lines supporting a direct role for SLFN11 in drug sensitivity. Then, they demonstrated that, in multiple patient-derived xenograft (PDX) models, SLFN11 expression assessed by IHC is associated with tumor response to talazoparib. Indeed, they generated an IHC SLFN11 score which ranges from 0 to 300 and integrates three intensities of IHC nuclear staining and their frequency (H-score). This IHC score proved to be a stronger predictor of PARP inhibitors (in particular, talazoparib) efficacy across these PDX lines than either SLFN11 gene expression, or protein expression by WB. Moreover, they showed that temozolomide(TMZ)—recently added to the NCCN guidelines for SCLC second-line therapy—is strongly synergistic with PARP inhibitors in vitro demonstrating also combinatorial efficacy in vivo. These data demonstrated that the role of

SLFN11-dependent drug sensitivity extends beyond conventional DDA to a targeted agent, becoming a relevant predictive biomarker of sensitivity to PARP inhibitors in SCLC. [21]

At the same time, Murai et al. (2016) reported the similar conclusion of Lok et al. concerning the potential role of SLFN11 expression as a dominant biomarker to predict response to PARP inhibitors as single agent acting by trapping PARP and damaging DNA (talazoparib, olaparib, and probably niraparib and rucaparib), as well as for combination regimens of broad PARP inhibitors with TMZ. Indeed, they showed that SCLC cell lines presenting high SLFN11 expression had more sensitivity to PARP inhibitors and SLFN11 transcript levels were significantly correlated to the IC50 of talazoparib. Furthermore, SLFN11 protein levels in the SCLC cell lines were measured by WB and, then they were matched with SLFN11 transcripts demonstrating the close relationship between the expression of mRNA and protein. [20]

Gardner et al. (2017) described that SFLN11 suppression is associated with acquired chemoresistance across independent SCLC models (mouse xenografts) and in primary human tumor samples. They demonstrated that overexpression of SLFN11 in chemo-resistant human cell lines restore sensitivity to topoisomerase poisons, indicating that SLFN11 expression directly contributes to chemosensitivity. Furthermore, SCLC cell lines generated from treated patients had lower levels of SLFN11 expression relative to lines generated from untreated patients. The authors assessed, on clinically annotated tumor microarrays from untreated (Vanderbilt Medical Center) and previously treated (Case Western Reserve University) SCLC patients, SLFN11 expression by IHC score (H-Score) and they found that was higher in tumors from patients who responded to therapy versus those who did not. Moreover, the same authors investigated whether EZH2, which is often highly expressed in SCLC, plays a role in regulating SLFN11 because its binding sites are upstream of SLFN11. They showed that EZH2 is induced by cytotoxic CT, resulting in deposition of repressive chromatin marks in the SLFN11 gene body, decreasing SLFN 11 expression and promoting chemo-resistance in SCLC. Finally, SLFN11 gene expression can be restored by pharmacological

inhibition of EZH2, even in the presence of DDA. So, they showed that SLFN11 is both necessary and sufficient for sensitivity to DDA in SCLC and they recognized that EZH2 inhibition increased expression of SLFN11 improving the response to CT. [55]

Stewart et al. characterized SCLC-specific biomarkers of therapeutic vulnerability, performing a high-throughput, integrated proteomic, transcriptomic, and genomic analysis using SCLC PDX models, cell lines, and archival tumor specimens. They found that the levels of SLFN11 expression determined response to both PARP inhibitors and several classes of CT in preclinical models. Indeed, in SCLC PDX models they found by reverse-phase protein array (RPPA) and RNA sequencing (RNAseq) that high protein expression of SLFN11 predicts the response to talazoparib. Furthermore, in a panel of untreated 51 SCLC cell lines, SLFN11 protein expression was the strongest marker of sensitivity with several CT (alkylating agents, cisplatin, TOP1 inhibitors, TOP2A/B inhibitors, and DNA synthesis inhibitors) and PARP inhibitors (talazoparib, $P<0.0001$; olaparib, $P=0.02$). Furthermore, treatment with cisplatin and both PARP inhibitors reduced SLFN11 levels (measured by WB) in cell lines having high endogenous expression of SLFN11. So, SFLN11 should also be used as a poor predictive biomarker of response to second or third line therapies. As with the cell lines, they also observed a bimodal distribution (high vs low expression) of SLFN11 in publicly available mRNA data from 70 early-stage, treatment-naïve SCLC patient tumors and in order to better understand the functional role of SLFN11 they performed Ingenuity Pathway Analysis comparing genes associated with high vs. low SLFN11 levels. Interestingly, these analyses revealed an enrichment of immune regulatory pathways, primarily interferon (IFN) signaling ($P=6.6*10-6$), in SLFN11-high tumors. Furthermore, they screened a curated gene list enriched for immune targets and found that high SLFN11 expression was positively correlated with PDL1 (CD274; rho=0.248, P=0.025), CCL2 (rho=0.271, P=0.014), CTLA4 (rho=0.221, P=0.046), and IL6 (rho=0.226, P=0.041) suggesting also a role of SLFN 11 in immune system. Finally, their findings support that expression of SLFN 11 as a biomarker to response to PARP inhibitors and CT is dynamically regulated by drugs used in frontline therapy. In addition, their results also show that

the dependence of SLFN11 levels on PARP1 may represent a novel mechanism of acquired resistance to PARP inhibitors. [22]

Pietanza et al., first showed that, in their randomized, double-blind clinical trial, SFLN11 expression was a predictive biomarker of chemo-response and progression-free survival (PFS) and overall survival (OS). Indeed, they showed that, in a pre-specified subgroup of their analysis, the patients included in the TMZ plus veliparib arm with SLFN11-positive tumours, as defined using IHC, had improved PFS (5.7 months versus 3.6 months; $P = 0.009$) and OS (12.2 months versus 7.5 months; $P = 0.014$) relative to patients with SLFN11-negative tumours. So, high SLFN11 expression could represent a predictive biomarker of response to PARP inhibitors in select SLCL patients. [56]

iv) Assessment of SFLN11

In the literature, the assessment of SFLN11 has been performed on three different levels: gene expression, mRNA expression, and protein expression.

Different studies confirmed the linear relationship among mRNA measured by qRT-PCR and protein expression (WB and IHC) derived by activation of SLFN 11 gene. [20,56]

In IHC and WB, several antibodies and different scores (Table N.1) have been used to evaluate SLFN 11 protein expression but nobody has been clearly validated. [17,21,54]

Provider and product name	*Source*	*Studies*	*WB, Dl*	*IHC, Dl*	*IHC score*	*IMF*
NBP1-92368 NB	Rabbit Poly	1) Li M et al. 2012*	1) Yes, N.S	1) No		1) No
HPA-023030 SA	Rabbit Poly	1) Barretina J et al. 2012 2) Lok BH et al. 2017 3) Gardner EE et al. 2017 4) Stewart CJR el al. 2017 5) Pietanza MC et al. 2018	1) Yes, 1:500 2) No 3) No 4) No 5) No	1) No 2) Yes, n.s. 3) Yes, N.S 4) Yes, 1:50 5) Yes N.S	1) No 2) Yes[I] 3) Yes[II] 4) Yes[III] 5) Yes[IV]	1) No 2) No 3) No 4) No 5) No
SC-136891 (K-13)	Goat Poly	1) Zoppoli G et al. 2012 2) Tian et al. 2014	1) Yes, 1:500 2) Yes, N.S	1) No 2) No		1) Yes 2) No
SC-374339 (E-4)	Mouse Mono	1) Abdel-Mohsen M et al. 2013 2) Tang SW et al. 2015 3) Kang MH et al. 2015 4) Goss Kl et al. 2016 5) Murai J et al. 2016 6) Nogales V et al. 2016	1) Yes, 1:500 2) Yes, N.S. 3) Yes, N.S 4) Yes, 1:500 5) Yes, N.S 6) Yes, N.S	1) No 2) No 3) No 4) No 5) No 6) No		1) No 2) No 3) No 4) No 5) No 6) Yes

		7) He T et al. 2017 8) Lok BH et al. 2017 9) Gardner EE et al. 2017 10) Tang SW et al. 2018 11) Valdez F et al. 2018*	7) Yes, 1:200 8) Yes, 1:250 9) Yes, 1:250 10) Yes 11) Yes, 1:500	7) No 8) No 9) No 10) No 11) No		7) No 8) No 9) No 10) No 11) Yes, 1:200
Ab-121731	Rabbit Poly	1) Deng Y et al. 2017	1) No	1) Yes	I+P (0-6)$	1) No
SC- 515071 (D-2)	Mouse Mono	1) Tang SW et al. 2018 2) Murai J et al. 2018** 3) Valdez F et al. 2018	1) Yes& 2) Yes, 1:10000 3) Yes, 1:500	1) No		1) No 2) Yes,1 :1000 3) Yes, 1:200

Table N. 1: Anti-Schlafen 11Antibodies

*: Studies in infectious disease (HIV-virus)

**: Immunoprecipitation (IP) and IP coupled to Mass Spectrometry: Anti-Schlafen11 antibody SC- 374339 (E-4)

Ab: Abcam, Dl: dilution, IHC: immunohistochemistry, IMF: Immunofluorescence, Mono: Monoclonal, NB: Novus biological, N.S: not specified, Poly: Polyclonal, SA: Sigma-Aldrich, SC: Santa Cruz, WB: western Blot,

$: Intensity was scored as 0, none; 1, weak; 2: moderate; 3, strong. The proportion of positive tumor cells was assigned to 0 (<25 % positive), 1 (25–50 % positive), 2 (50–75 % positive), 3 (75–100 % positive). The final score was calculated by intensity plus proportion (0–6).

&: In this paper the authors don't clearly describe how and when they used two different antibodies

[I]: Three intensities of IHC nuclear staining and their frequency. A final expression (H-score) from 0 to 300

[II]: The same score of Lok et al. (2017)

[III]: Nuclear expression of SLFN11 were quantified using a 4-value intensity score (0, none; 1, weak; 2, moderate; and 3, strong) and the percentage (0%–100%) of the extent of reactivity. A final expression score (H-score) was obtained by multiplying the intensity and reactivity extension values (range, 0–300) as described previously [3, 4].

[IV]: Sections were scored for intensity (0-3+) and extent (0- 100%) of staining by light microscopy. By multiplying intensity and extent of staining, each tumor was assigned an H-score (range 0-300). For SLFN11, an immunohistochemistry (IHC) score of 1 or greater was considered positive.

The following link https://www.proteinatlas.org/ENSG00000172716-SLFN11describes:

1) SLFN 11 expression in the normal and pathologic tissue.
2) Localization of SLFN 11 in the cell
3) Information about a gene, mRNA/protein expression and state of validation of antibodies

Today, a reliable and validate protocol to assess IHC SLFN 11 expression in vitro, in vivo and in-patient tumors has not been clearly defined not allowing to translate these finding to a clinical setting. Indeed, in the literature, different type of antibodies, several dilutions of antibody and different IHC scores had been described and a common protocol should be created by different laboratory groups.

v) Function of SLFN11

The precise mechanism by which SLFN11 sensitizes cancer cells to DNA-targeted agents is not fully established.

a) Action of SLFN11 as executioner of replication stress

SLFN11 was discovered by bioinformatics analyses of cancer cell databases as a dominant determinant of response to widely used anticancer drugs (Top 1 and 2, Alkylating agents and DNA synthesis inhibitors) and, moreover, a link between high SLFN11 expression and hypersensitivity to PARP inhibitors has recently been established. Interestingly, a common mechanism of action among these drugs is DNA damage leading to replication fork stalling with cell cycle checkpoint activation, also referred to as replication stress that should be, therefore, considered the common mechanism(s) engaging SLFN11 to kill cancer cells.

Several and recent studies have investigated the molecular functions of SLFN11. [17,20,57,58]

The first molecular connection between SLFN11 and replication stress was the co-immunoprecipitation of SLFN11 with replication protein A (RPA)1, a replication and repair protein binding to ssDNA generated by replication stress and DNA excision prior to homologous recombination and nucleotide excision repair. Mu et al. confirmed that SLFN11 interacts directly with RPA1 and revealed that SLFN11 is recruited to end-resected DNA lesions via RPA1 after DDA. [58] They proposed that SLFN11 inhibits checkpoint maintenance and homologous

recombination repair by promoting the destabilization of the RPA-ssDNA complex, thereby sensing cancer cell lines with high SLFN11 to DDA. [58]

First Zoppoli et al. (2012) and, then, Murai et al. (2016 and 2018) showed that SLFN11 induces lethal replication block in response to a broad type of DNA-targeting agents. Moreover, Murai et al showed that SLFN11 induces lethal replication block in response to PARP inhibitors independently of ATR and proposed that SLFN11 acts in parallel with the ATR-mediated S-phase checkpoint. So, SLFN11-mediated cell cycle arrest is permanent and lethal while the ATR-CHEK1-mediated S-phase checkpoint is transient and enables cell survival. [20] Murai et al. recently elucidated some key molecular mechanisms by which SLFN11 irreversibly blocks replication. [57] Figure N. 9

1)Under normal conditions, replication forks only form short ssDNA segments coated with RPA, and SLFN11 does not gain access and does not block replication progression, explaining why SLFN11 does not interfere with normal replication.

2) Upon replication damage and stress, RPA filaments are generated on single-stranded DNA both at resected DNA ends and at stressed replication forks. DNA breaks activate ATR and slow down replication, leading to the uncoupling of the MCM helicase complex and DNA polymerases at stressed replicons (Figure N.9, pathway 1). Activated ATR and CHK1 halt replication initiation by inhibiting the loading of CDC45 (Figure N.9, pathway 2). SLFN11 binds both resected DNA ends and stressed replication forks via RPA1, where it interacts with MCM3, opens chromatin, and blocks replication (Figure N.9, pathway 1). As SLFN11 does not inhibit replication initiation, stressed replication forks with RPA filaments are generated, recruiting SLFN11, which blocks fork progression (Figure N.9). Moreover, they demonstrate that the ATPase activity of SLFN11 is not required for the recruitment of SLFN11 to chromatin but is required to block fork progression and to open chromatin. Although the mechanisms of replication block by the ATPase activity of SLFN11 are not fully understood, a plausible scenario is that once SLFN11 binds stressed

replication forks, chromatin becomes open in a SLFN11-dependent process ahead of the MCM helicase, which blocks the MCM complex and fork progression.

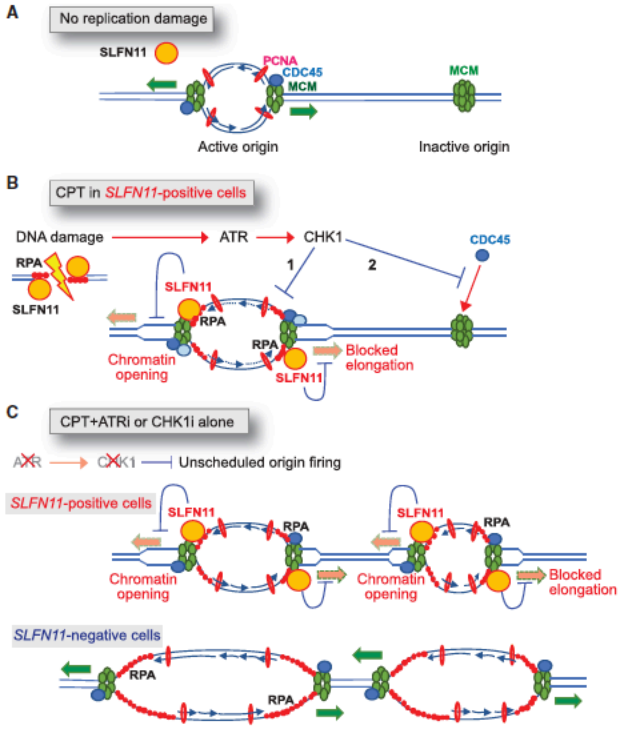


Figure N.9 Molecular Model of SLFN11- Induced Replication Fork Block in Response to Replication-Stress

A) Replication without replicative stress. B) Replication stress induced by CPT in *SLFN11*- positive cells. C) Unscheduled origin firing induced by CPT+ATR inhibitor or by CHK1 inhibitor in *SLFN11*-positive (top) and *SLFN11*-negative (bottom) cells

b) Regulation of SLFN11 expression

Interestingly, SLFN11 is inactivated at the transcription level in approximately half of the cell lines across the available cancer cell line databases, including the NCI-60[19] the CCLE[16], and the Genomics of Drug Sensitivity in Cancer project (GDSC) (Yang et al., 2013) defining its expression bimodal, i.e. cells either express or do not express SLFN11. [16] Moreover, a high correlation between protein expression and gene expression is showed in several studies. [17,20,56]Because whole exome sequencing and copy number analyses of the NCI-60 showed that, the SLFN11 non-expressing cells had no detectable copy loss or deleterious mutation (Varma S PLOS 2014), the expression of SLFN11 should be regulated in three different ways: 1) epigenetically (promoter

methylation); 2) transcriptionally (ETS transcription factor binding); and 3) in response to viral infections (IFN signaling).

1) Today, several epigenetic mechanisms seem to control SLFN11 expression: hyper/methylation of CpG promoter island[19,59], chromatin condensation by EZH2, acetylation by histone deacetylase (HDAC) inhibitors[60] and chromatin methylation by the polycomb repressor complex (PRC). [22] Concerning the methylation of *SLFN11,* it is among the genes with the highest correlation (at the top 94th percentile) between methylation and expression across over 1000 cancer cell lines including the NCI-60 and the Sanger-Mass General cancer cell lines. Moreover, among the cells lacking SLFN11 expression, promoter hypermethylation occurs in approximately half of the cases and his methylation is significantly correlated with resistance to different DDA. [19,61] He et al. showed that SLFN11 is methylated in 56% of primary CRC samples and that the methylation of the SLFN11 gene should be used as a marker of poor prognosis and platinum resistance in CRC. [53] SLFN11 hypermethylation was an independent prognostic factor in patients with non-SCLC and OC who received platinum-based chemotherapy. In both tumors, SLFN11 hypermethylation was significantly associated with shorter progression-free survival. [17] Resistance to cisplatin or PARP inhibitors in SCLC is associated with silencing of SLFN11 caused by EZH2, a histone methyltransferase targeting H3K27me3 inducing deposition of repressive chromatin marks in the SLFN11 gene body, and by the catalytic component of PRC2. [22] Indeed, EZH2 inhibition prevents the acquisition of chemo-resistance and improves CT efficacy in SCLC.[55]

2) EWS, which is characterized by translocations generating the chimeric transcription factor EWS-FLI1, presented the highest SLFN11 expression among 4103 primary tumor samples in CCLE. [16] One of mechanisms of transcriptional activation in EWS is the binding of EWS-FLI1 at ETS consensus sites in the SLFN11 promoter. The correlated expression

between SLFN11 and *FLI1* extends to other tumors as leukemia, pediatric, colon, breast, and prostate cancers. [41]

3) SLFN11 is induced by IFN-b, poly-inosine-cytosine or poly dAdT (Li, et al., 2012). SLFN11 expression is also significantly correlated with 16 Type I IFN signaling pathway genes and targetable immune markers PDL1 and CTLA4 in treatment-naïve early-stage SCLC patient tumors. [22] Thus, SLFN11 is likely to contribute to anti-viral and native immune functions. The mechanisms proposed for the antiviral activity of SLFN11 is its binding to tRNA, which specifically abrogates the production of retroviruses such HIV-1 by selectively inhibiting, at late stage, the expression of viral proteins in a codon usage-dependent manner. [12]

vi) SLFN11, Immune Response and Immunotherapy

Immunotherapy is the treatment of a disease by inducing, enhancing or suppressing an immune response.

In the oncological field, immunotherapy can be active or passive. Active immunotherapy directs the immune system to attack tumor cells by targeting tumor-associated antigens. In the other side, passive immunotherapy enhances existing anti-tumor responses and include the use of monoclonal antibodies, lymphocyte, and cytokines, including IFN.

IFNs play an essential role in innate immunity and in immune surveillance against cancers. [62] Extensive work over the years has shown that Type I IFNs bind to specific cell surface receptors and activate receptor-associated Jak kinases that engage the Stat pathways. [63]

Recent works have established that mouse and human several SLFN genes and corresponding proteins are induced in response to engagement of the human Type I IFN receptor[3,10,11] and are thus classified as Interferon-Stimulated Genes that possess anti-viral ability, growth inhibitory and antineoplastic effects.[7,12] Treatment of mouse cells with IFNa strongly induced mRNA expression for several SLFN proteins, including Slfn1, Slfn2, Slfn5, and Slfn8. It was shown that Stat1 was required for induction of all IFN-inducible mouse Slfn genes, the p38 MAPK was also required. [7] Similarly, there is evidence that human SLFNs are induced in response to engagement of the human Type I IFN receptor. Remarkably, mRNA expression for all human SLFNs studied, including SLFN5, SLFN11, SLFN12, and SLFN13, was induced in normal melanocytes, while only SLFN5 was inducible in melanoma cell lines. [11] There is accumulating evidence that mouse and human SLFNs have important roles in the generation of IFN-inducible response.

Moreover, the expression of most SLFN genes is abundant in immune cells and alters during the development of immune cells participating in the modulation of the immune system. Precisely, SLFN1, SLFN2, SLFN8, SLFN12, and SLFN12L are the members that have been reported to modulate T cell activation, macrophage differentiation, and monocyte maturation. [9]

The members of human SLFN are regulated differently during the process of differentiation from monocytes to dendritic cells (DCs). SLFN11 is expressed highly in the unstimulated monocytes and DCs, suggesting that it may play an important role in regulating the function of monocytes and DC cells. When monocytes are induced into DCs, the expressions of SLFN12L and SLFN13 increase substantially, which suggest that they might be an influencing factor in cell differentiation. In contrast, the level of SLFN12 is downregulated, which suggests that it may play a negative role in the process

of DC differentiation. To the contrary, SLFN12 is upregulated during the activation of T cells.[10]

In our unpublished data, we found in high grade serous OC(HGSOC) and BC positive correlation between SLFN11 and immune system. Precisely SLFN11 is strongly correlated with a subgroup of BC patients characterized by activation of immune markers and features partially overlapping with triple negative BC, such as low expression of hormone receptors and absence of HER2 overexpression. In HGSOC, SLFN11 expression showed significantly correlation with CD8+ intratumoral lymphocytes. Finally, SLFN11 could be expressed by immune cells during the anti-tumoral response, potentially behaving as a marker of T-cell (CD3+ and CD8+).

Stewart et al. found a correlation of SLFN11 with several immune-related targets as 16 Type I IFN signaling pathway genes and targetable immune markers PDL1 and CTLA4 in early-stage SCLC. [22] Li et al. showed that SLFN11 is expressed by T-cells and monocytes and it is an IFN- stimulated gene in peripheral blood mononuclear cells.[12]

Finally, SLFN11 could also be a potential surrogate marker of T-cell or macrophage infiltration and a biomarker for response to immunotherapy drugs targeting PDL1 and CTLA4, but this requires further testing in a clinic setting.

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3)Article type: Meeting report

Title page

Title: Report on the first SLFN11 monothematic workshop: from function to role as a biomarker in cancer

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Abstract: SLFN11 is a recently discovered protein with a putative DNA/RNA helicase function. First identified in association with the maturation of thymocytes, SLFN11 was later causally associated, by two independent groups, with the resistance to DNA damaging agents such as topoisomerase I and II inhibitors, platinum compounds, and other alkylators, making it an attractive molecule for biomarker development. Later, SLFN11 was linked to antiviral response in human cells and interferon production, establishing a potential bond between immunity and chemotherapy. Recently, we demonstrated the potential role of SLN11 as a biomarker to predict sensitivity to the carboplatin/taxol combination in ovarian cancer. The present manuscript reports on the first international monothematic workshop on SLFN11. Several researchers from around the world, directly and actively involved in the discovery, functional characterization, and study of SLFN11 for its biomarker and medicinal properties gathered to share their views on the current knowledge advances concerning SLFN11. The aim of the manuscript is to summarize the authors' interventions and the main take-home messages resulting from the workshop.

Keywords (up to 10): SLFN11, biomarker, immune system, DNA damage repair, chemotherapy, prognosis, prediction, ovarian cancer, colorectal cancer, breast cancer

Main text

Introduction: SLFN11 potential as a predictive biomarker to assess response to DNA damage inhibitors.

Presented by Gabriele Zoppoli

SLFN11 is a putative DNA/RNA helicase, first described for its role in thymocyte development and differentiation in mouse models [1]. SLFN11 is part of a family of proteins with various degree of homology across species, but intriguingly being consistently present only in vertebrates and especially in mammals (Fig. 1). The helicase domain is present only in the "long" SLFN proteins such as SLFN11, whereas the "short" SLFN proteins share only a domain of unknown function (the SLFN domain); finally, the intermediate SLFN proteins also possess a highly conserved SWADL motif, but lack the helicase domain [2]. Recently, while correlating the in vitro activity of topoisomerase I (TOP1) inhibitors with the transcriptional profiles of more than 20,000 genes in the NCI-60 cancer cell line model, we discovered by serendipity an unusually strict association between the levels of SLFN11 and the sensitivity to such DNA damaging agents (DDA). Subsequently, we observed that such high correlation was maintained with TOP2 inhibitors, as well as alkylating agents such as cisplatin. We then corroborated our discovery by modulation of SLFN11 expression in lung, colon, breast, and prostate cancer cell lines (HOP-62, HCT-116, MDA-MB-231, and DU-145 respectively), thereby demonstrating the causal relationship between SLFN11 intracellular levels and sensitivity to DDA [3]. Independently, Barretina and co-authors reported that Ewing's sarcoma cell lines had very high SLFN11. In line with our findings, those authors also described the tight correlation between SLFN11 transcript expression and TOP1 inhibitor toxicity in cancer cells [4]. In parallel with the findings concerning SLFN11 in cancer, its role and relation with the immune system, as well as the property of behaving as an early interferon-response gene were described [5]. Taken together, the published data points toward a possible connection between SLFN11, immunity and cancer. Indeed, it was not long since scientific

reports appeared, describing SLFN11 as a biomarker of response to DDA in human cancer. Moreover, since no evident mutations or copy number variations of SLFN11 could be find in cell models or in patients' cohorts such as the cancer genome atlas (TCGA), researchers have focused their attention on SLFN11 regulation by methylation. Indeed, SLFN11 hypermethylation is associated with worse prognosis in ovarian cancer and with a poor response to platinum derived compounds in lung cancer [6]; consistently we have observed that SLFN11 overexpression purports a platinum-sensitive phenotype in patients affected by such neoplasm. More recently, SLFN11 has been associated with sensitivity to PARP inhibitors and other DDA in both cancer models and in clinical case sets. In conclusion, SLFN11 appears as a promising molecule both for its causative implication in sensitivity to DDA, as a biomarker of response to such agents, and for its potential as a link between immunity, cancer, and response to chemotherapy.

Cell cycle inhibitory function of SLFN11 in the DNA damage response (DDR).

Presented by Elisabetta Leo

SLFN11 was recently identified as a novel DNA damage response (DDR) gene in cancer cell genomic analyses of the NCI60 [3] and the cancer cell line encyclopedia (CCLE) [4] cancer cell models. In 2012 we reported the causative effect of SLFN11 as a determinant of cancer cell sensitivity to multiple DNA damaging agents in different human cell lines: upon downregulation of SLFN11 by siRNA, cells showed a dramatic increase in viability after short and long-term treatments with camptothecin (CPT) and other DNA damaging agents (DDA) when compared to SLFN11-proficient cell lines [3]. After these initial observations, we worked to elucidate the mechanisms by which SLFN11 impinges on the DDR.

We found that, in SLFN11 proficient cells, SLFN11 protein levels are constant in all the phases of the cell cycle (after FACS-sorting as well as upon pharmacological synchronization) G1, S, G2 and mitosis. Subcellular fractionation studies revealed that SLFN11 is preferentially localized in the nuclear compartment, and binds tighter to the chromatin upon accumulation of DNA damage.

SLFN11 in the nucleus forms foci that are in close proximity ahead of the replication foci. SLFN11 is preferentially present in the euchromatic regions (open chromatin, identified by H3K9Ac) and is clearly excluded from heterochromatin (H3K9Me3). We also examined cell cycle progression and DNA replication after CPT treatment in SLFN11-proficient versus SLFN11-down- regulated cells. In non-treated cells there is no apparent phenotypic difference; however, during treatment with low doses of CPT, dramatic differences in cell cycle and in DDR are observed between SLFN11-proficient and -deficient cells; these differences are visible as early as 4 h after DDA-treatments [7]. If cells are SLFN11 proficient, they undergo an enforced G1/S arrest, with tight cell cycle and replication block, which leads to cell death [8, 9]. On the contrary, if SLFN11 is absent, cells are capable to re-enter the cell cycle, slowly progress through S-phase and are less prone to die. This slow progression is associated to an hyper-activation of the DNA replication and damage checkpoint: indeed, very high and persistent phosphorylation of ATM, ATR, Chk1, an Chk2 are observed. When SLFN11-depleted cells are co-treated with CPT and either ATM, ATR or Chk1/2 inhibitors, they progress much faster through S-phase and they are ultimately re-sensitized to the damage.

We suggest that SLFN11 works as an additional cell cycle checkpoint, that possibly acts upstream of the classical replication and damage checkpoint, preventing the cells to progress and to survive when they accumulate DNA damage and replication stress [7]. Based on these observations, we can conclude that SLFN11 has high potential relevance in the clinics as predictive biomarker for patient stratification. SLFN11-proficient tumors may be more likely to respond to a DDA-based chemotherapy, whereas SLFN11-deficient tumors might require more aggressive combination treatments, for example with ATM or ATR inhibitors, or different anticancer strategies.

SLFN11 induces lethal S-phase arrest in response to DNA damage—a novel mechanism of how cancer cells are killed by DNA damaging agents.

Presented by Yves Pommier and Junko Murai

In two works previously published in 2012 and 2014, our group reported the founding of a novel mechanism of action for PARP inhibitors named PARP-trapping, which explains why PARP inhibitors act as DNA damaging agents [10, 11]. PARP inhibitors trap PARP1 and PARP2 at DNA single strand break lesions, which are common and highly cytotoxic because of inducing replication stress. The potency of PARP trapping is widely different among clinical PARP inhibitors, and talazoparib is the most potent PARP trapping inhibitor so far. We reported that sensitivity profile of talazoparib in NCI-60 is highly correlated with the expression profile of SLFN11. The correlation was shown to be causal using four isogenic cell lines (parental cells with high SLFN11 expression vs their SLFN11-knockout cells) and extended to other PARP inhibitors including olaparib, and the combination of talazoparib and temozolomide. Although deficiency of homologous recombination is a dominant determinant of hypersensitivity to PARP inhibitors, SLFN11 sensitized cells in a parallel pathway with homologous recombination deficiency. SLFN11 induced irreversible and lethal S-phase arrest under continuous talazoparib treatment for 48 h, while cells without SLFN11 slowly reached G2-phase and viable at that time under the regulation of S-phase checkpoint by ATR activation. The abrogation of S-phase checkpoint by the addition of ATR inhibitor (ATRi) with PARP inhibitors, which enforces unscheduled origin firing, synergized cells drastically. Hence, we propose two distinct strategies to kill cancer cells (Fig. 2) using PARP inhibitors; one is to induce SLFN11- dependent replication arrest by PARP inhibitor alone or the combination with temozolomide, the other is to use PARP inhibitors with ATR inhibitors to induce lethal unscheduled origin firing in SLFN11-deficient cells [12].

Predictive markers in ovarian cancer

Presented by Domenico Ferraioli

Ovarian cancer is the seventh most common cancer worldwide and the eighth cause of cancer death in women [13]. Early stages are hard to detect, and several patients are diagnosed when the disease is already in an advanced stage [14]. Standard recommendations for patients with advanced ovarian cancer (AOC) include primary debulking surgery (PDS) followed by platinum-based adjuvant chemotherapy [15] but, in some cases, the PDS is not feasible or is associated with unacceptable morbidity; therefore, neoadjuvant chemotherapy (NACT) followed by debulking surgery should be performed [16]. Patient response to chemotherapy for ovarian cancer is extremely heterogeneous and approximately 60% of patients with AOC will relapse after first-line chemo- therapy [17]. Nowadays, tools predicting the sensitivity or the resistance to chemotherapy and allowing treatment stratification are not available; nevertheless, different biomarker assays are in active development. These approaches include functional assays, identification of resistance gene markers, and micro RNA analysis. A systematic review of 42 studies concerning the prediction of chemotherapy response in AOC using gene expression was performed in 2015 by Lloyd et al. [18]. The authors concluded that a clinically applicable gene signature can- not be identified, highlighting the presence of a severe heterogeneity concerning the histological type, the tis- sue preservation techniques applied, and the manners of obtaining the gene signature among the different studies. Chemoresponse tests, or other biomarker assays, are thus not recommended to choose a chemotherapy regimen. The majority of the available studies failed to demonstrate a survival benefit of chemotherapy regimens selected on chemoresponse assays compared to chemotherapy regimens selected using traditional clinical factors [15]. To conclude, a validated predictive biomarker does not currently exist, and the international guidelines only suggest the use of CA 125 to monitor response to chemotherapy as part of a clinical trial [19]. Well-designed randomized controlled trials are needed to develop a predictive model of response to chemotherapy.

SLFN11 assessment in ovarian cancer: phenotypic and histological distribution and association with TIL infiltration

Presented by Valerio Gaetano Vellone.

Epithelial carcinoma of the ovary has always been clinically considered as one disease, but there is now a much greater realization that the various subtypes have a different natural behavior and prognosis [20]. At present, adjuvant therapy is mainly dependent upon tumor stage and grade rather than type [15]. However, it is of common observation how tumors with similar stage and histologic type can behave in radical different ways and finding potential molecular markers represents one of the challenges of modern surgical pathology. To date, DNA-damaging chemotherapeutic agents constitute the backbone of treatment for most solid and hematological tumors. High expression levels of SLFN11 seems to correlate with the sensitivity of human cancer cells to DNA- damaging agents [3]. In this setting, it appears clear how immunohistochemistry (IHC) testing for SLFN11 may represent a powerful tool to predict the response and modulate the chemotherapy for high-grade serous ovarian carcinoma (HGSC). To date no commercial kit for SLFN11 IHC testing is available, so we adapted two kits originally commercialized for Western Blot (WB), and we tested a population of 75 cases of HGSC. As positive control, we used a commercial culture of ovarian carcinoma (SKOV-3) processed with agarose-embedded cell block technique (CCB); SKOV-3 cell culture is known to have high level of expression for SLFN11.

In some cells we observed a crescent-shaped thickening of the coloration in the perinuclear area consistent with Golgi complex (Fig. 3A). No staining was observed in the nuclei. This observation is in apparent contrast with what was reported by Zoppoli et al. [3] and by Dr E. Leo in their work. However, nuclear antigens may translocate into cytoplasm or dispersed by nuclear wall disruption upon either apoptonecrotic processes intervening during tissue exeresis or due to the fixation processes. Hence, we cannot currently conclude that SLFN11 staining in formalin fixed, paraffin-embedded cells reflects SLFN11 location in living tissues. It has to be noted that, although Dr Leo's subcellular fractionation showed a preferential nuclear localization, a proportion of SLFN11 was

also present in the cytoplasm. Furthermore, at least two publications have pointed out that SLFN11 may be found also in cytoplasm, so currently we can only speculate that this protein may translocate following active processes in living/dying cells as well [1, 21].

Immunohistochemistry appeared clean and specific, no aspecific bonds were observed in tumor and residual ovarian stroma (Fig. 3B), and a relevant positive internal control was represented by a subpopulation of tumor infiltrating lymphocytes (TIL), which stained intensively for SLFN11 (Fig. 3C). SLFN11 expression resulted extremely variable among cases, and even in different fields of the same tumor. However, a dominant pattern of intensity seems to exist in the same neoplasia. For each case we assessed both the intensity score (IS) and the distribution score (DS) evaluating at least 300 cells. Intensity score (IS) evaluates the main pattern of intensity of stain in positive cancer cells as follow: 0: no stain (Fig. 3C); 1 ±: weak stain (visible at high magnification) (Fig. 3D); 2 +: moderate stain (visible at scan magnification) (Fig. 3E); 3 +: intense stain (Fig. 3F). Distribution score (DS) evaluates the percentage of stained cancer cells as follow: 0: no stained cells; 1 +: < 10% of stained cells; 2 +: 10–40% of stained cells; 3 +: > 40% of stained cells. These scores were combined to obtain a final histological score (HS) as follow: HS = IS × DS. Study cases were grouped on the base of HS in the following categories: cases with HS = 0 were considered SFLN11 negative, cases with HS 1 and 2 were considered SFLN11 low, cases with HS 3 and 4 were considered SFLN11 intermediate, while cases with HS 6 and 9 were considered SFLN11 high. At the end of the evaluation, the SLFN11 expression in the studied case set was distributed with an elegant Gaussian-like fashion: 27 cases (39.13%) resulted SLFN11 negative, 11 cases (15.94%) resulted SLFN11 low, 23 cases (33.33%) resulted SLFN11 intermediate and 8 cases (11.59%) resulted SLFN11 high. Globally, SLFN11 appears to be poorly expressed in HGSC, with the larger subpopulation com- posed by cases with no sign of stain (SLFN11 negative). We hypothesize that, if SLFN11 negative cases mirror the large population of chemotherapy-resistant patients, SLFN11-high cases may identify a subpopulation of chemotherapy-responsive patients with a better prognosis. Of interest, only a subpopulation of TIL appears to express SLFN11. Their nature and

biological role remain to be studied. In the future the presented IHC data will be matched with RNA expression data and clinical data such as overall survival and disease-free interval, to better estimate the role of SLFN11 as a potential novel, pivotal prognostic marker in HGSC.

Molecular determinants of immune responsiveness in breast cancer and putative role of SLFN11.

Presented by Davide Bedognetti

By exploiting the integrative data available from the cancer genome atlas, we assessed the determinants of immune response in breast cancer (BC) [22]. In that work, we identified that a T helper cell phenotype upregulation is associated with a better prognosis, validating such observation in an independent data set [23]. SLFN11 was discovered in association with thymocyte maturation [1], and appears as an interferon (IFN) regulated gene [5]. To investigate the transcriptional land-scape of SLFN11 in BC, we performed a gene expression microarray meta-analysis of more than 7000 cases from 35 publicly available data sets [24]. By pan-transcriptional SLFN11 correlative analysis, we identified 537 transcripts in the top 95th percentile of Pearson's coefficients with SLFN11. The terms "lymphocyte activation", "immune response", and "T cell activation" resulted as top gene ontology enriched processes [25]. We lever- aged the method of multiple corresponding analysis, a multivariate statistical process aimed at inferring mutual associations among categorical variables [26]. Thus, we identified a patient cluster defined by elevated SLFN11 expression, ER lack of staining, basal-like PAM50 phenotype, increased CD3D, STAT1 signature [25], and younger age at diagnosis. By penalized maximum likelihood lasso regression [27], we observed a very strong association of SLFN11 with the previously described stroma 1 and stroma 2 signatures [28, 29]. These signatures usually appear upregulated in basal-like BC and in ER- tumors responding to chemotherapy. Finally, using Cox proportional hazard regression, we characterized SLFN11 high levels, high proliferation index, and ER negativity as independent parameters for

longer disease- free interval in patients undergoing chemotherapy. Alto- gether, our data point toward a role for SLFN11 in BC, in likely connection with the immune system modulation in such disease entity.

SLFN11 and sensitivity to irinotecan in colon cancer.

Presented by Sana Intidhar Labidi-Galy

SLFN11 has recently been identified as the protein with the highest correlation with sensitivity to topoisomerase I inhibitors such as irinotecan in the NCI60 cancer cell lines [3] and in the cancer cell line encyclopedia [4]. We investigated the correlation between the expression of SLFN11 and survival in colon cancer patients treated in the PETACC3 study, a randomized phase III trial that included 3278 patients in the adjuvant setting and compared two regimens of chemotherapy: half of the patients received LV5-FU2 regimen (5-FU based chemo- therapy) while the other half received FOLFIRI regimen (LV5-FU2 and irinotecan). No significant improvement in disease-free survival (DFS) or overall survival (OS) was detected by adding irinotecan to LV5-FU2 as adju- vant therapy [30]. Patients' tumor samples were collected and gene expression profile analysis was performed on 553 tumors [31]. In the FOLFIRI regimen group (285 patients), we surprisingly observed that patients with SLFN11-high tumors manifested a worse outcome than those having SLFN11-low tumors (7 years-OS 70.6% vs 79.3%, HR = 1.53, 95% CI 1.012–2.503, Log-Rank $p = 0.044$), while in the LV5-FU2 group (268 patients who received only LV5-FU2 regimen) SLFN11 levels did not have any impact on survival (7 years-OS 71.6% vs 73.0%, HR = 1.034, 95% CI 0.667–1.603, $p = 0.88$). We then investigated the interaction between SLFN11 levels and microsatellite instability (MSI) status [32], observing a trend toward increased levels of SLFN11 in MSI-high tumors (40/64 = 62.5%) compared to micro- satellite stable (MSS) tumors (244/489 = 49.89%, Fisher test $p = 0.06$). We divided the patients into four groups: group 1 (MSI-high and SLFN11-high), group 2 (MSI- high and SLFN11-low), group 3 (MSS and SLFN11-high) and group 4 (MSS and SLFN11-

low). In the LV5-FU2 group, there was absolutely no difference whether tumors were SLFN11-high/low, MSI or MSS tumors (Fig. 4a); in the FOLFIRI group, we observed that among tumors with MSI-high—having a very high rates of mutation [33]—the patients with SLFN11-high tumors showed a better outcome compared to those having MSI-High but SLFN11-low tumors (7 years-OS 95% vs 66.7%, HR = 0.129, 95% CI 0.014–1.156, p = 0.067). Inversely, in patients with MSS tumors, we observed a worse outcome in patient SLFN11-high than in SLFN11-low (7 years- OS 64.6% vs 82.1%, HR = 2.348, 95% CI 1.412–3.904, p = 0.001) (Fig. 4b). Analyzing these data in a multivariable model, we demonstrated that the interaction between MSI status and SLFN11 was significant (p = 0.011). One study addressed the prognostic significance of SLFN11 overexpression in colorectal (CRC) cancers. The cohort included 261 patients with stage II or III CRC cancers treated with oxaliplatin-based adjuvant chemotherapy. SLFN11 was assessed by immunohistochemistry [34].

Overall, CRC with high SLFN11 levels did not show prolonged survival. A substantial benefit from SLFN11 overexpression was observed only in the sub-group of patients with *KRAS* wild-type tumors. SLFN11 overexpression did not have impact on the outcome of patients harboring somatic *KRAS* mutation (exon 2). There is an overlap between MSI and *KRAS* status, with 90% of CRC MSI-high being *KRAS* wild-type ($p < 0.001$) [35]. Together, these observations suggest that a subgroup of CRC tumors MSI-high, *KRAS* wild-type, overexpressing SLFN11, is very likely to benefit from DDA-based adjuvant chemotherapy. In the future, it would be interesting to better identify this sub-group of tumors and investigate at the molecular level the mechanisms underlying such benefit.

Consensus conclusions

Shared by all the co-authors

1. SLFN11 is a protein with a causal association with response to DDA in cancer cells.
2. SLFN11 is induced by IFN, but the current relation- ship between TILs and SLFN11 expression in cancer tissues is not known.
3. SLFN11 can be assessed in human cancer tissues by IHC, with wide range of expression.
4. Several preclinical and clinical models point toward SLFN11 as a predictive marker of response to DDA and PARP inhibitors.
5. SLFN11 expression may be related to mutational burden and MSI in colon cancer.
6. At present, the predictive role of SLFN11 expression in human tumors is unclear and needs further investigation.
7. At present, there is no consensus on the exact function of SLFN11 in health and disease, but all available evidence points toward its relevance in cancer.

Authors' contributions

All authors equally contributed to writing the present manuscript, revised it. All authors read and approved the final manuscript.

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Figure titles and Legends

Figure 1: Conservation tree of SLFN11 across species. Constructed using the Ensembl! GeneTree tool, queried with the term "SLFN11" (last accessed 2017, June 19).

Figure 2: Summary scheme proposing the role of SLFN11 in parallel to ATR and homologous recombination [12].

Figure 3: Immunohistochemistry staining for SLFN11: **A** (IHC; 400×) Positive external control constituted by SKOV-3 cell block culture. **B** (IHC; 400×) Negative external control constituded by normal menopausal ovary: no stain in both overian surface cells and stromal cells. **C** (IHC, 400×) SLFN negative HGSC: cancer cells show no stain, TILs show an intense stain representing a useful internal control. **D** (IHC, 400×) SLFN low HGSC (HS 2) with a faint (IS 1 +) inconstant (DS 2 +) pattern of stain. **E** (IHC, 400×) SLFN intermediate HGSC (HS4) with a moderate (IS 2 +) inconstant (DS 2 +) pattern of stain. **F** (IHC, 400×) SLFN high HGSC (HS 6) with an intense (IS 3 +) but inconstant (DS 2 +) pattern of stain

Figure 4: Overall survival in patients of the PETACC3 study according to SLFN11 levels and MSI status. A) Overall survival in the 268 patients treated with LV5-FU2 regimen. B) Overall survival in the 285 patients treated with FOLFIRI regimen.

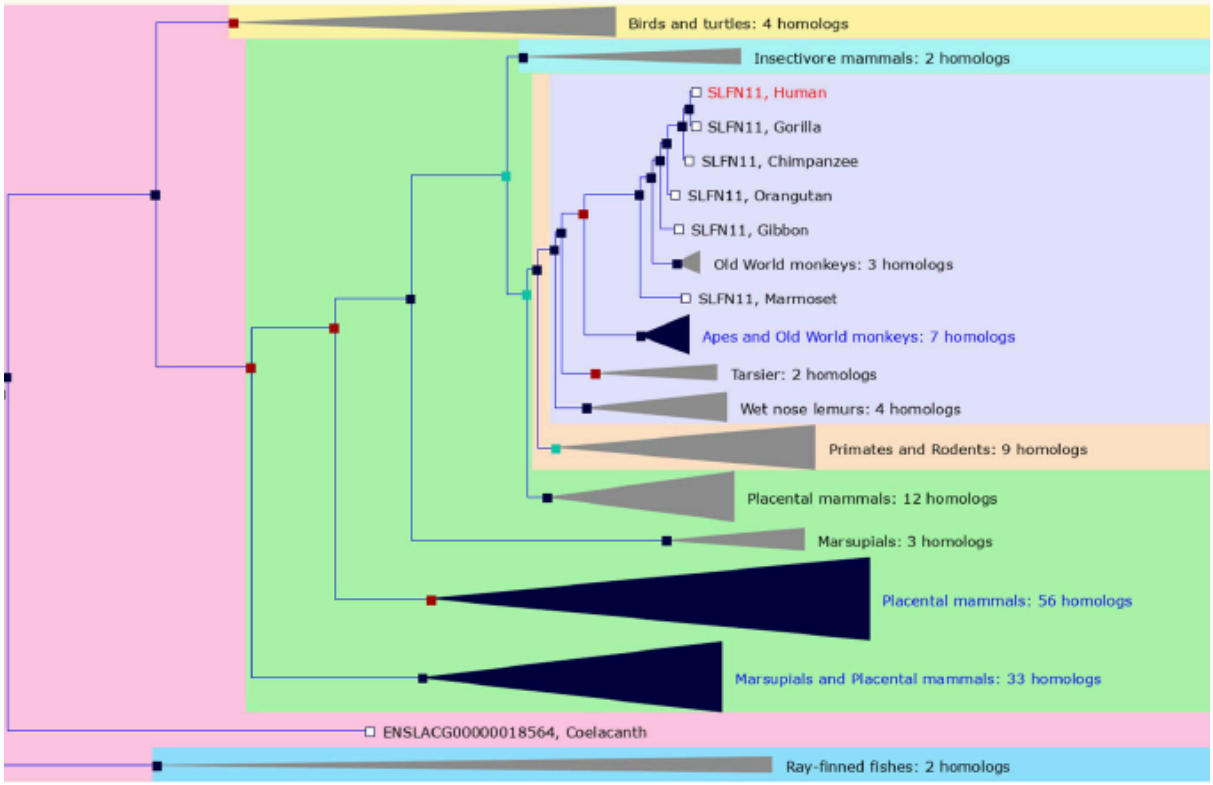
Birds and turtles: 4 homologs
Insectivore mammals: 2 homologs
SLFN11, Human
SLFN11, Gorilla
SLFN11, Chimpanzee
SLFN11, Orangutan
SLFN11, Gibbon
Old World monkeys: 3 homologs
SLFN11, Marmoset
Apes and Old World monkeys: 7 homologs
Tarsier: 2 homologs
Wet nose lemurs: 4 homologs
Primates and Rodents: 9 homologs
Placental mammals: 12 homologs
Marsupials: 3 homologs
Placental mammals: 56 homologs
Marsupials and Placental mammals: 33 homologs
ENSLACG00000018564, Coelacanth
Ray-finned fishes: 2 homologs

Figure 1

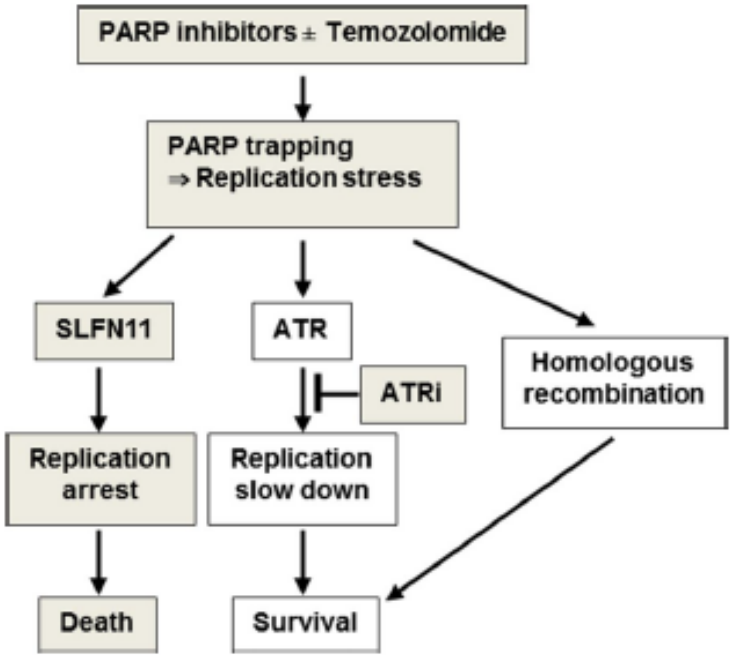

Figure 2

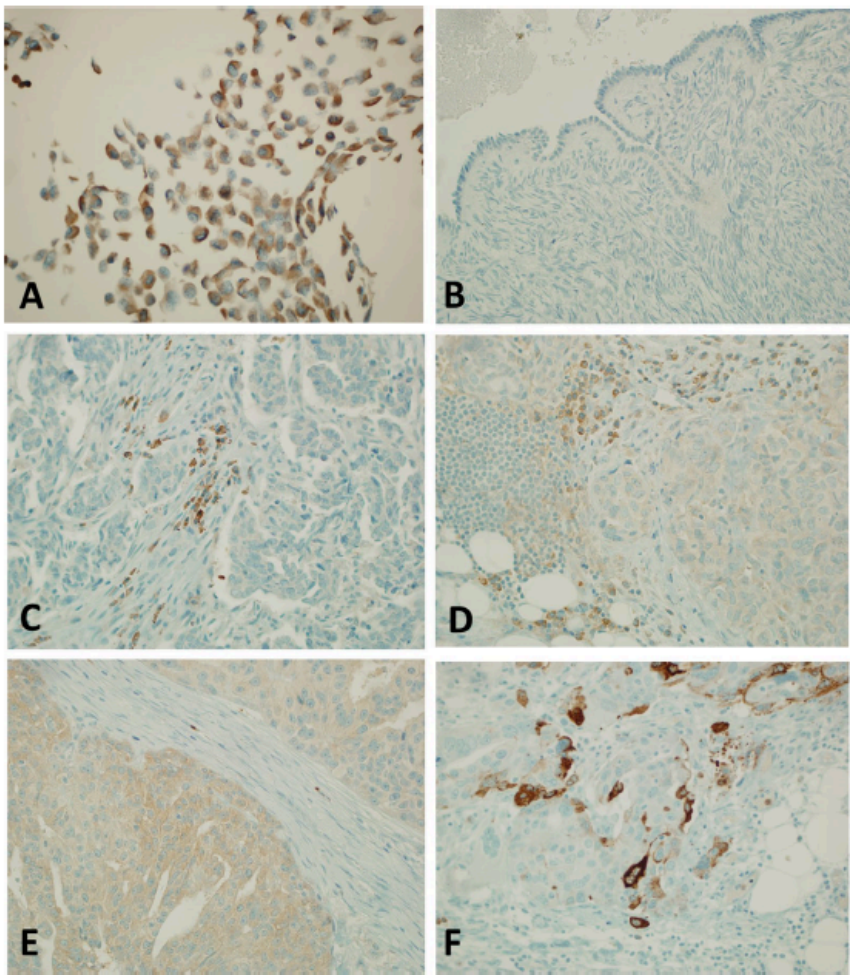

Figure 3

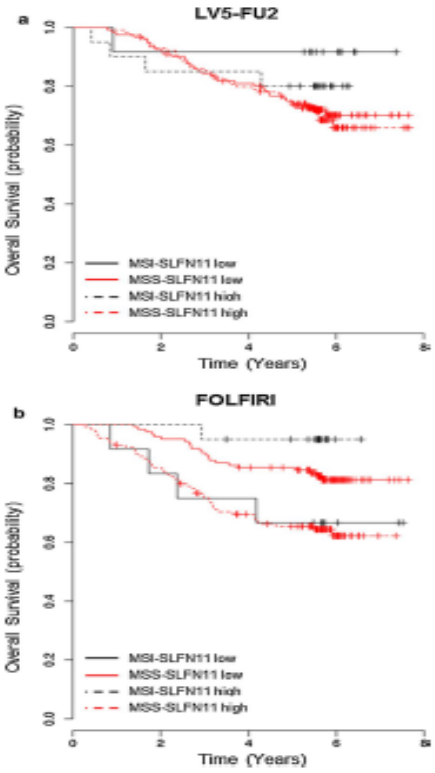
a
LV5-FU2
Overall Survival (probability)
0.0
0.2
0.4
0.6
0.8
1.0
MSI-SLFN11 low
MSS-SLFN11 low
MSI-SLFN11 high
MSS-SLFN11 high
0
2
4
6
8
Time (Years)
b
FOLFIRI
Overall Survival (probability)
0.0
0.2
0.4
0.6
0.8
1.0
MSI-SLFN11 low
MSS-SLFN11 low
MSI-SLFN11 high
MSS-SLFN11 high
0
2
4
6
8
Time (Years)

Figure 4

4) ***Article type:*** Research Article

Full title:

Schlafen-11 assessment in high grade serous ovarian carcinoma: phenotypic and histological distribution

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ABSTRACT:

The aim of our study was to develop a reliable protocol for immunohistochemistry (IHC) in order to determine Schlafen-11 (SFLN11) expression in formalin-fixed paraffin-embedded (FFPE) high-grade serous ovarian carcinoma (HGSOCs) samples.

Firstly, we validated a reliable SLFN 11 antibody (Ab) in IHC choosing between two antiSLFN11-Abs previously tested for Western blot (WB) through the development of a SFLN11-IHC protocol in culture cell block (CCB) of ovarian carcinoma (OC) and in an HGSOCs tissue micro-array (TMA) series. Successively, we applied our protocol to a case series of HGSOC samples to confirm our preliminary results. For each case, we evaluated both the Intensity Score (IS) and the Distribution Score (DS). A final Histological Score (HS) was obtained as follow: HS=IS x DS

We found that in CCB and TMA series, Ab #1 at 1:100 dilution was more reliable and we decided to use this last one in our case series to confirm our IHC protocol.

DS showed the following results: 27 cases were not stained, 11 cases showed staining for SFLN11 in <10% of tumour cells, 16 cases showed staining in 10-40% of cells and the remaining 15 cases showed stain in >40% of cells.

IS showed the following results: 25 cases were not stained, 19 cases had a mostly weak stain, 14 cases a moderate stain, and 11 cases showed a strong stain.

HS for SLFN11 expression presents a normal distribution with a prevalent (≈ 60%) intermediate expression.

In summary, we developed a reproducible and standardized IHC protocol to determine SFLN11 protein expression in FFPE HGSOC samples using a modified WB anti SLFN11 Ab.

Key words:

Schlafen-11, SLFN11, Serous High-Grade Ovarian Cancer, Immunohistochemistry, Histological Score, Anti-SLFN11 antibody

Introduction:

Ovarian cancer (OC) is the seventh most common cancer and the eighth cause of death from cancer in women.[1] The incidence of OC increases with age and is most prevalent in the sixth and seventh decades of life.[2] OC is a highly heterogeneous group of diseases, including different histological subtypes with distinct clinico-pathological and molecular features, and are generally classified as Type I and Type II tumours.[3] Among them, high-grade serous ovarian carcinoma (HGSOC) is the most common histologic subtype of ovarian cancer, accounting for three quarters of ovarian carcinoma.[4-7]

Nearly 70-80% of HGSOC are diagnosed in advanced stage, when peritoneal carcinomatosis or distant metastases are established (International Federation of Gynecology and Obstetrics, FIGO stages III-IV).[8]

Primary debulking surgery (PDS) followed by systemic platinum-based chemotherapy (CT) is the standard of care.[9]

It has been observed that although 40%-60% of patients achieve complete clinical response to first-line CT treatment[10], around 50% of these patients relapse within 5 years[11], and only 10%-15% of patients presenting with advanced stage disease achieve long-term remission.[11] It is thought that the high relapse rate is at least in part due to resistance to chemotherapy, which may be inherent or acquired by altered gene expression.[12]

Nowadays, no tools are available to predict chemotherapy response sensitivity in OC.

We recently discovered the role of a putative DNA/RNA helicase, Schlafen 11 (SLFN11), for its causal association with sensitivity to DNA damaging agents, such as platinum salts, topoisomerase I and II inhibitors, and other alkylators in the NCI-60 panel of cancer cell lines.[13]

SLFN11 belongs to the Schlafen protein family, which has been implicated in the regulation of important mammalian biological functions, such as control of cell proliferation[14], induction of immune responses[15], and regulation of viral replication.[16]

It is now thought that SLFN11 blocks replication by changing chromatin structure across replication

sites upon DNA damage.[17]

Recently, the importance of SLFN11 for drug sensitivity has recently been extended to Ewing's sarcomas, in ovarian, colorectal (CRC) and non-small cell lung cancers.[13,18-20]

SLFN11 was used as a predictive biomarker for nal-irinotecan sensitivity[21], furthermore, it inhibits checkpoint maintenance and homologous recombination by removing Replication Protein A from single stranded DNA.[22]

SLFN11 expression by immunohistochemistry (IHC) staining was correlated directly with survival and other clinico-pathological factors in CRC, and could be considered as both a prognostic and a predictive factor in CRC KRAS wild-type subgroup. [23]

IHC is a powerful tool in surgical pathology. Amongst its several advantages, IHC is relatively cheap, it is available in most histopathology facilities worldwide, it allows for direct morphological-molecular correlations, and can be performed on archived paraffin-embedded sections. There is an increasingly focused attention on IHC for its capability in identifying potential factors of susceptibility to chemotherapy.

The purpose of this study was to develop a reliable protocol for SFLN11 IHC testing in formalin-fixed paraffin-embedded (FFPE) samples and to describe SLFN11 staining features in a large series of HGSOC cases.

Materials and Methods

Patients cohort

A multicentric retrospective study was realized between January 2014 and January 2017. A database of 199 HGSOC patients was analysed. The clinical characteristics, the histo-pathological features and types of treatment were recorded.

Eligibility criteria were as follows: a) Diagnosis of HGSOC, b) Stage III-IV according to FIGO classification at diagnosis, c) Performance status 0-1 at diagnosis, d) Surgical biopsy before medical treatment, e) Menopausal status, f) Written informed consent to the use of cancer specimens for

cancer research, in agreement with the Local Ethical Committee of Cancer Center Léon Bérard, University of Geneva, and Ospedale Policlinico San Martino. All researches were conducted according to the principles of the Helsinki Declaration.

Histo-pathological Analysis

We developed our work over two phases: 1) In the first one, we tested and validated a reliable SLFN 11 antibody (Ab) for IHC developing a SFLN11-IHC staining protocol; 2) subsequently, we applied our protocol to HGSOC samples meeting our eligibility criteria in order to confirm our preliminary results.

Cell Cultures Block (CCB)

As a positive control and to test which of the two Anti-SLFN11 antibodies was the most reliable for IHC testing, we used a cancer cell line of ovarian carcinoma, SKOV-3, that has endogenous high levels of expression for SFLN11[13] (gift of Dr. Anne Monks, National Institutes of Health, Bethesda MD). In brief, SKOV-3 cultured cells were trypsinized, washed with Tris Buffered Saline (TBS), fixed with 10% formalin and pre-embedded in 3% agarose to CCBs as described previously.[2]

Tissue Micro-Arrays (TMA)

In order to choose the most reliable anti-SFLN11 Ab and test the feasibility of our SLFN11-IHC protocol, a senior pathologist (VGV) with long-standing experience in HGSOC evaluation performed a centralized analysis on 12 independent HGSOC specimens from ovarian masses, not included in the case series. A total of five 4-mm wide tissue cores were obtained from these 12 cases of HGSOC, coming from three different FFPE blocks and representative of the whole carcinoma. Necrotic or poorly fixed areas were excluded from evaluation. Additionally, four specimens of normal ovarian tissue from healthy donors were used as negative control. For recipient blocks, we used two 6x5 Matrices of Tissue-tek® Quick-Ray (Manufacturer: Sakura Finetek USA, Inc) following the Manufacturer's specifications.

An FFPE block, representative of the entire tumour, was chosen for SLFN11 IHC testing, and to prepare TMA paraffin blocks from each of these specimens.

Tissue Processing

All the ovarian HGSOC specimens were fixed in buffered formalin for 12-18h, routinely processed, embedded in paraffin to obtain 3-µm thick histological slides and mounted on positively charged Superfrost Plus® Slides.

Antibodies

We chose two SLFN11 antibodies for Western blot (WB) commercial kits for IHC testing, since at the beginning of our study no IHC-optimized anti-SLFN 11 antibodies were commercially available for use in FFPE material (see Figure N.I).

The first one was a Slfn11 (D-12) affinity-purified goat polyclonal Ab raised against a peptide mapping within an internal region of Slfn11 of human origin marketed by Santa Cruz [Slfn11 (D-12): sc-136890; Santa Cruz Biotechnology, Inc.; Dallas, TX, USA]. Slfn11 (D-12) was recommended for detection of Slfn11 of human origin by WB, immunofluorescence, and solid phase ELISA. The D-12 Ab was marketed as non cross-reactive with other SLFN family members. (SC-136890, Santa Cruz Biotechnology, https://www.scbt.com/scbt/fr/product/slfn11-antibody-d-12)

The second one was a SLFN11 purified mouse monoclonal Ab in phosphate buffer saline with 0.05% sodium azide, marketed by Merck-Millipore [Anti-SLFN11, clone 4G9; Cat # MABF248; Lot # Q2430677; EMD Millipore Corporation, Temecula, USA]. Anti-SLFN11 Ab, clone 4G9 is an Ab against SLFN11 for use in WB and immunoprecipitation. (http://www.merckmillipore.com/FR/fr/product/Anti-SLFN11-Antibody-clone-4G9,MM_NF-MABF248)

Since no pre-existing dilution indication was known for the used SLFN11 Ab for IHC, we had to

test several dilutions to determine which was the optimal one. Both anti-SLFN11 Abs were therefore tested at 1:25; 1:50 and 1:100 dilutions.

Successively, we evaluated both staining patterns and differences in intensity for different cellular and tissue components.

IHC Testing

For IHC testing we used an automatic immunostainer Benchmark XT (Ventana Medical Systems SA, Strasbourg, France). CB and TMA slides were tested with D12 and 4G9 anti-SFLN11 antibodies at 1:25; 1:50, and 1:100 dilutions. Antigen retrieval was obtained with citrate buffer (pH 6) at 90°C for 30 minutes. Then, samples were incubated with primary Abs for 1 hour at 37°C, followed by the addition of the polymeric detection system Ventana Medical System Ultraview Universal DAB Detection Kit, counterstained with modified Gill's hematoxylin and mounted in Eukitt. All TMA slides were processed during the same session. Slides form our multicentric case series were tested in a total three sessions in different days. Each staining session included an external positive control (SKOV-3 CCB) and an external negative control (no primary Ab in four cases of healthy ovarian tissue) (see Figure N. II A and B).

IHC Evaluation

Complete IHC testing was evaluated by the same senior pathologist (VGV). D-12 and 4G9 anti-SFLN11 antibodies were tested and compared to determinate which one was more reliable to use in the case series.

For each case, both the Intensity Score (IS) and the Distribution Score (DS) were evaluated in at least 300 cells.

Intensity Score (IS) assesses the main pattern of staining intensity in positive cancer cells as follows:

0: no stain

1+: weak stain (visible at high magnification)

2+: moderate stain (visible at scan magnification)

3+: intense stain (Tumor Infiltrating Lymphocytes –TIL)

Distribution Score (DS) evaluates the percentage of stained cancer cells as follow:

0: no stained cells

1+: <10% of stained cells

2+: 10-40% of stained cells

3+: >40% of stained cells

The scores were combined to obtain a final ***Histological Score (HS)*** as follow: ***HS=IS x DS***.

Study cases were grouped based on HS within the following categories: cases with HS=0 were considered SFLN11 Negative, cases with $0<HS\leq 2$ were considered SFLN11 Low, cases with $2<HS<6$ were considered SFLN11 Intermediate, while cases with $HS\geq 6$ were considered SFLN11 High.

Concerning TMAs IS, DS, and HS were separately evaluated for each tissue core. Then, for each case a final mean IS, DS and HS was calculated. Based on the final mean HS, each case was assigned to the proper SFLN11 category: SFLN11 Absent, SFLN11 Low, SFLN11 Intermediate, and SFLN11 High.

Results

Cell Culture Block (CCB), **Figure N. IIB**

CCB stained with Ab #1 (D-12) showed intense and diffuse cytoplasmic stain. well discernible up to 1:100 dilution. In some cells, a crescent-shaped thickening of the coloration in the perinuclear area consistent with Golgi complex was also observed. No staining was observed in the nuclei (Figure N.II and N.III). Ab #2 (4G9) showed a paler and diffuse stain and even the nuclei appeared weakly stained.

TMAs

Being clearly visible with the lowest concentration, TMAs were tested in independent specimens with Ab#1 at 1:100 concentration to avoid any possible non-specific bond. SFLN11 IHC with Ab #1 appeared clean and specific, and no non-specific staining could be appreciated in the tumour stroma (Figure N. IIA). A relevant positive internal control was represented by a subpopulation of tumor infiltrating lymphocytes (TIL) with intensive stain (3+) for SFLN11, in agreement with the literature[25] (Figure N. III-Right,F). No staining was observed on ovarian coelomic epithelium nor in ovarian inclusion cysts and albicans bodies (Figure N. IIA).

IHC staining for SFLN11 with Ab #1 was extremely variable between cases (inter-tumor staining heterogeneity), and even within the same cases (intra-tumor staining heterogeneity); (Figure N.IV). Staining distribution was highly heterogeneous even in the same case, varying from few scattered stained cells (DS=1) (Figure N. III-Right,H) to a diffuse, homogeneous staining (DS=3); (Figure N. III-Right,F). Likewise, staining intensity per se, even in homogeneous samples, exhibited a wide range, from a faint stain visible at high magnification (IS=1) (Figure N. III-Right,F) to an intense staining similar to that previously observed in control CB (IS=3); (Figure N.III-Right,H).However, a predominant intensity pattern seems to characterize individual cases.

Combining IS and DS to obtain HS, 3 cases resulted SFLN11 Negative, 5 cases were SLFLN11 Low, 3 cases were SFLN11 Intermediate and one case SFLN11 High (see Figure N. IV).

Ab #2 was judged unreliable, as it repeatedly failed to reveal the striking differences between and within cases observed with Ab #1. Moreover, all the tested dilutions (1:25; 1:50 and 1:100) showed a diffuse, faint stain on cancer cells, which became stronger only using high antibody concentration at the likely cost of a lower binding specificity. At the same concentration, all the independent series showed the same diffuse pattern of staining with minimal or no differences in intensity.

For these reasons, we decided to perform IHC of our case series with Ab #1 (Santa Cruz D-12) at 1:100 dilution.

Case Series

70 HGSOC samples were eligible for this study. One case was excluded due to insufficient cancer cell fraction. The remaining 69 cases underwent IHC testing for SFLN11 using Ab #1 at 1:100 dilution. Four cases of healthy ovarian tissue were used as a negative control (see Figure N.IIA).

This large case series confirmed our preliminary observations performed on TMAs.

Distribution Score (DS) showed the following results: 27 cases (39.1%) showed no stain (0+), 11 cases (15.9%) showed staining for SFLN-11 in <10% of tumour cells (1+), 16 cases (23.2%) showed staining in 10-40% of cells (2+) and the remaining 15 cases (21.7%) showed stain in >40% of cells (3+).

Intensity Score (IS) showed the following results: in 25 cases (36.2%) we observed no stain (0+), in 19 cases (27.5%) a dominant weak stain (1+), in 14 cases (23.2%) a moderate stain (2+), and in 11 cases (15.9) a strong stain.

DS and IS were combined to obtain HS and divide the study population in the proposed categories. As observed in Figure N.V, when SLFN11 was expressed (~ 60% of cases), it showed a normal distribution with more prevalent intermediate expression (H3 and H4). In contrast, SLFN11 was not expressed (H0) in 40% of cases. This lack of expression was related with both negative DS and IS.

Discussion

There are scarce literature examples concerning the use of anti-SLFN11 Abs for IHC in cancer specimens. Deng *et al.* studied the correlation between IHC expression of SLFN 11 in CRC and overall survival. They used a rabbit polyclonal anti SLFN11-Ab produced by Abcam Company at a dilution of 1:50[26] that is normally used for WB, immunocytochemistry and immunofluorescence. Lok *et al.* evaluated IHC expression of SLFN11 on PDX model using a non-conjugated polyclonal rabbit Anti-SLFN11 Ab produced by Sigma-Aldrich but they did not specify if they tested our Ab in FFPE samples. [27]

In none of these works a systematic, analytically designed procedure for FFPE staining of SLFN11

by IHC was reported. In our study, we aimed to develop and validate a SLFN11 Ab for use in a reproducible and standardized IHC procedure to determinate the SFLN11 expression in FFPE samples.

In the first phase of our endeavour, we tested by IHC two different, promising SFLN11-Abs previously used for other SLFN11 detection purposes. We did so by: a) staining a positive control, the SKOV-3 cell culture, that presents naturally high SLFN11 levels, and b) testing our two candidate Ab in 12 independent HGSOC specimens.

The preparation of CCB and TMAs reproduces the normal procedure that we use during the process of fixation of a biopsy from any tissue for FFPE embedding. For antigen retrieval, we chose to exploit the most commonly used protocol in daily practice, automating the procedure with a widely available commercial autostainer.

Already by IHC staining of CBs, Ab #1 (Santa Cruz clone D-12) showed greater reliability than Ab #2 (Merck-Millipore clone G49). These results were confirmed in the independent HGSOC sample series. Indeed, by using the same antibody concentrations used in clinical practice, Ab #1 produced meaningful, clear results. Not only it allowed us to observe an intra-tumoral heterogeneity for SLFN11 staining, but it successfully stratified or case series into 4 clearly subdivided subgroups ranging from faint to intense staining. Furthermore, SFLN11 IHC staining performed with Ab # 1 appeared to be reliable and significant for the presence of a unique internal control represented by TIL and for the absence of non-specific staining of peri-tumoral stromal elements. Ab #2 failed at showing SLFN11 staining of TIL, which are known to express this protein[25], and presented with aspecific staining of external non-cellular elements.

Finally, Ab #2 failed to reproduce intra-tumor heterogeneity for SLFN11, and to stratify between different cases, resulting in a practical impossibility to differentiate between cases. Indeed, a same diffuse pattern of staining with minimal or no difference in intensity was seen in CB and in our independent series at all the concentrations tested.

SFLN11 appears to be variably expressed in HGSOC, with 27 out of 69 cases (39.1%) showing no

staining at all (SFLN11 Negative). In the remaining specimens, we observed an extremely wide range for SLFN11 expression, allowing us to differentiate samples into four different subpopulations that, in clinical practice, may purport a different response to chemotherapy.

Of extreme interest, 16 (59.3%) of the 27 SLFN11 Negative subpopulation were platinum-resistant according to recent literature data that highlighed the role of SLFN11 in response to DNA damaging agents (DDA). Indeed, low expression of SLFN11 may confer a resistance to DDA or PARP inhibitors, both widely used in HGSOC.[13,20,26-29]

We are aware of the limitations in our study. First, the used case set represents a multicentric, retrospective collection of samples, which is not the optimal starting point for the evaluation of a novel biomarker. On the other hand, cases were rigorously selected and underwent centralized reassessment by an experienced pathologist, ensuring that a correct diagnosis and proper sample conditions would be guaranteed. A second weak point in our analysis is the lack of clinical follow-up data. We are currently collecting information concerning our dataset as well as a prospective, larger series which should answer to the question of a correlation between SLFN11 IHC levels and prediction/prognosis in HGSOC. Finally, although *in vivo* analyses have shown that SLFN11 is mainly located in nuclei, the Ab we chose actually tended to stain the cytoplasmic reticulum and not the nuclear region. This however is not an indication of aspecific binding, since we showed that in SLFN11 expresser cancer cells, Ab #2 was able to exactly pinpoint the expression of that protein. Moreover, during ischemia and fixation processes several proteins could be extruded from nuclei and could be found, when assessed in FFPE material by IHC, in cellular cytoplasm. As a further proof-of-principle that SLFN11 staining was specific in our experience, we observed that TIL, known to express SLFN11 constitutes an internal positive control.

In conclusion, we were able to optimize a reproducible, valid, and standardized IHC procedure to determine SFLN11 expression in FFPE samples. Biological observations from our report, such as intra- and inter-tumor SLFN11 expression heterogeneity and TIL staining are intriguing and elicit several questions for future clinical and preclinical studies. We believe that our methodological

work can now pave the way for the use of SLFN11 IHC staining in human cancer samples to assess the role of this protein as a prognostic and predictive biomarker.

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Disclosure

The authors have declared no conflicts of interest.

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Figures and Figure Legends:

	Antibody #1	**Antibody #2**
Original application	WB, ELISA, IF	WB, IP
Species Cross-Reactivity	Human	Human
Antibody Isotype	IgG1	IgMK
Host Species	Goat	Mouse
Dilutions	1: 25	1: 25
	1: 50	1: 50
	1: 100	1: 100
Molecular Weight (kDa)	103	100

Figure N. I: Characteristics of two antibodies used to perform IHC

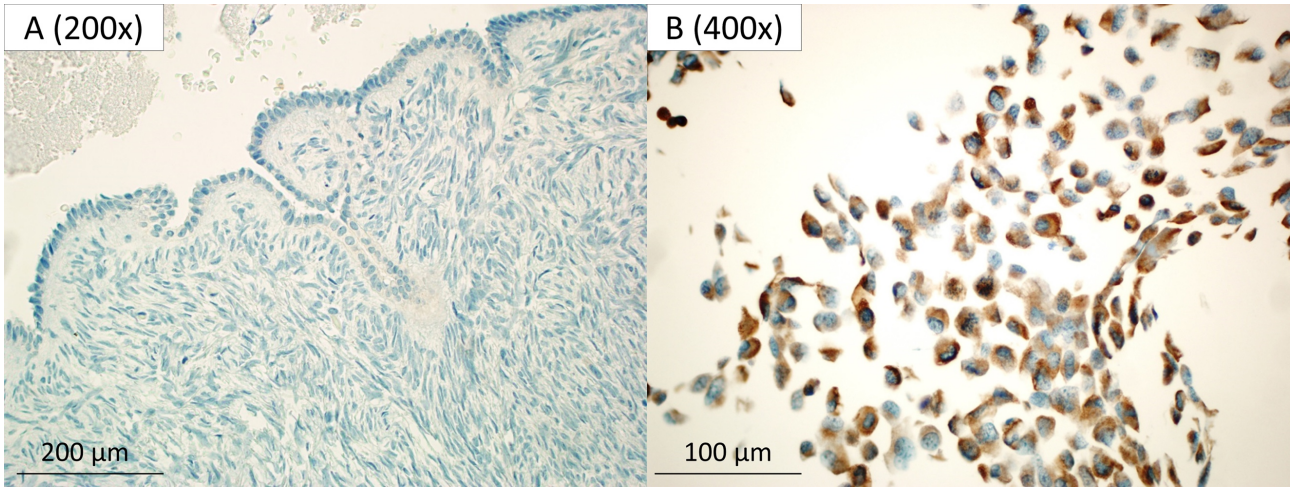


Figure N. II: ***A, (IHC; 200X):*** Healthy ovarian tissue (negative control), both ovarian surface and stroma are not stained by SFLN-11; ***B, (IHC; 400X):*** CCB (SKOV-3, positive control**)** stained with antibody #1 at 1:110 dilution, diffuse and intense staining (3+).

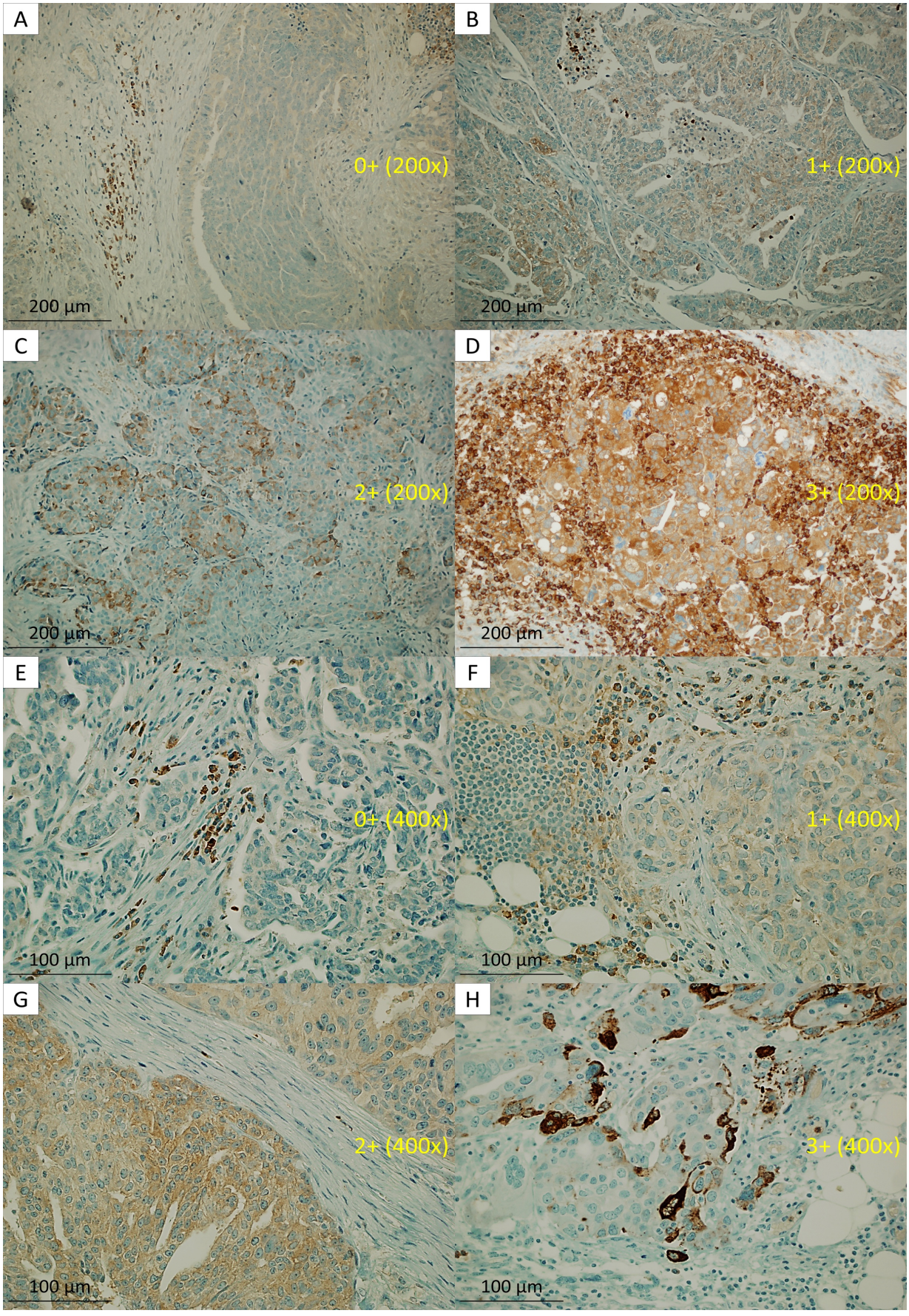
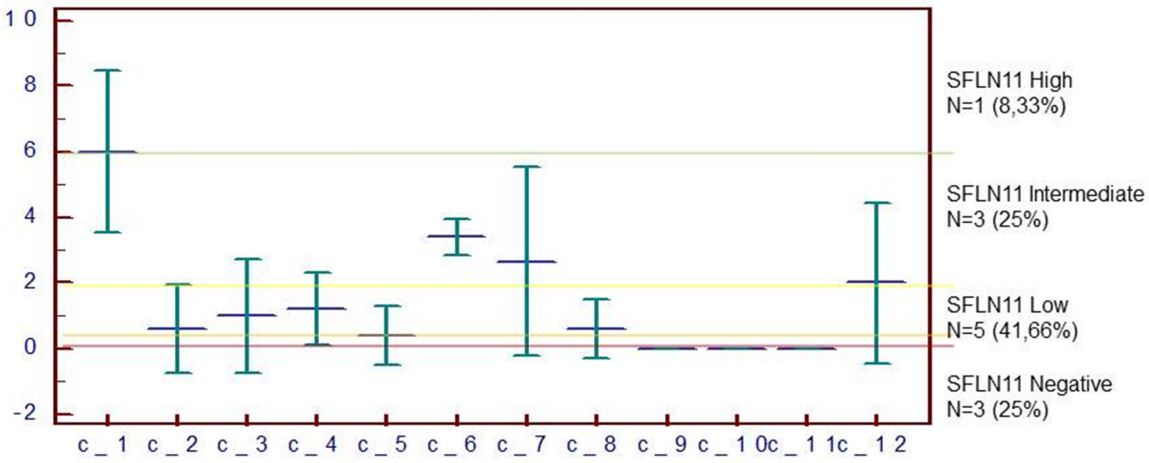

A
0+ (200x)
200 µm
B
1+ (200x)
200 µm
C
2+ (200x)
200 µm
D
3+ (200x)
200 µm
E
0+ (400x)
100 µm
F
1+ (400x)
100 µm
G
2+ (400x)
100 µm
H
3+ (400x)
100 µm

Figure N. III: Density Score (Left, A-D) and Intensity Score (Right, E-H) of SLFN 11 in IHC TMA series to validate the Ab



Case	# cores	Mean IS	SD	Mean DS	SD	Mean HS	SD	SFLN11 category
c_1	4	3,00	0,00	2,00	0,00	6,00	2,45	high
c_2	5	0,60	1,34	0,20	1,34	0,60	1,34	low
c_3	5	0,60	0,89	0,60	0,89	1,00	1,73	low
c_4	5	1,00	1,00	0,80	1,00	1,20	1,10	low
c_5	5	0,40	0,89	0,20	0,89	0,40	0,89	low
c_6	5	2,60	0,55	1,40	0,55	3,40	0,55	intermediate
c_7	5	1,33	0,58	1,67	0,58	2,67	2,71	intermediate
c_8	3	0,40	0,55	0,60	0,55	0,60	0,89	low
c_9	5	0,00	0,00	0,00	0,00	0,00	0,00	neg
c_10	5	0,00	0,00	0,00	0,00	0,00	0,00	neg
c_11	5	0,00	0,00	0,00	0,00	0,00	0,00	neg
c_12	5	1,00	1,00	1,20	1,00	2,00	2,45	intermediate

Figure N. IV: Histological Score of SLFN 11 in IHC TMA series; *Blue Bar*: TMAs Case Mean HS, *Green Line*: TMAs HS±1SD

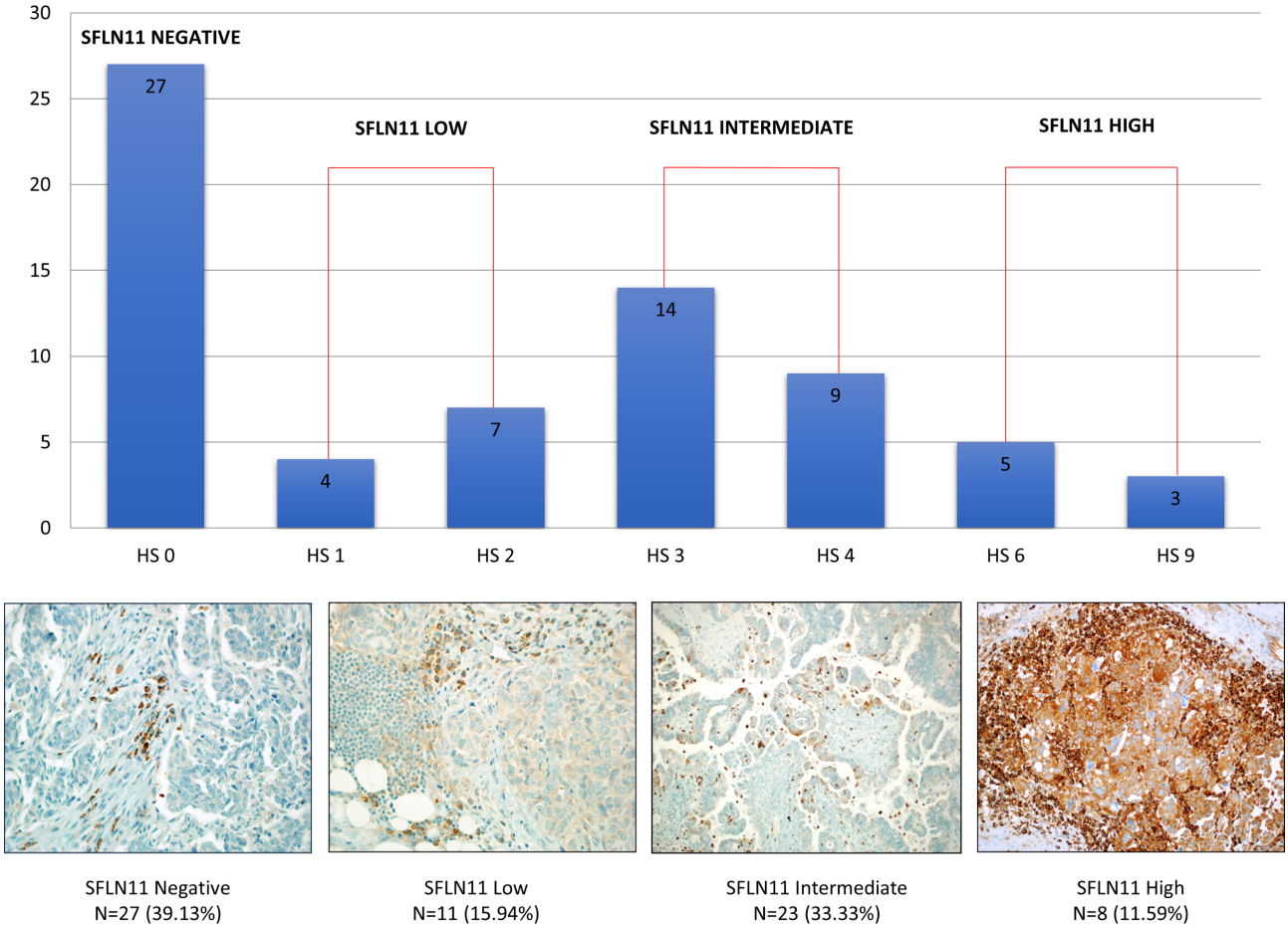


Figure N. V: Histological Score of SLFN 11 in IHC Case series (High); Correlation between Histological Score and IHC expression of SLFN 11(Low)

5) TITLE PAGE

Full Title: ***Schlafen-11 expression is associated with immune signatures and basal-like phenotype in breast cancer.***

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ABSTRACT

PURPOSE: Breast cancer (BC) is a heterogeneous disorder, with variable response to systemic chemotherapy. Likewise, BC shows highly complex immune activation patterns, only in part reflecting classical histopathological subtyping. Schlafen-11 (SLFN11) is a nuclear protein we independently described as causal factor of sensitivity to DNA damaging agents (DDA) in cancer cell line models. SLFN11 has been reported as a predictive biomarker for DDA and PARP inhibitors in human neoplasms. SLFN11 has been implicated in several immune processes such as thymocyte maturation and antiviral response through the activation of interferon signaling pathway, suggesting its potential relevance as a link between immunity and cancer. In the present work, we investigated the transcriptional landscape of SLFN11, its potential prognostic value, and the clinico-pathological associations with its variability in BC.

METHODS: We assessed SLFN11 determinants in a gene expression meta-set of 5,061 breast cancer patients annotated with clinical data and multigene signatures.

RESULTS: We found that 537 transcripts are highly correlated with SLFN11, identifying “immune response”, “lymphocyte activation”, and “T cell activation” as top Gene Ontology processes. We established a strong association of SLFN11 with stromal signatures of basal-like phenotype and response to chemotherapy in estrogen receptor negative (ER-) BC. We identified a distinct subgroup of patients, characterized by high SLFN11 levels, ER- status, basal-like phenotype, immune activation, and younger age. Finally, we observed an independent positive predictive role for SLFN11 in BC.

CONCLUSIONS:

Our findings are suggestive of a relevant role for SLFN11 in BC and its immune and molecular variability.

Keywords: Schlafen-11, Immune Signatures, Basal-like phenotype, Breast Cancer, Biomarker

List of abbreviations

BC: breast cancer

DDA: DNA damaging agents

DFS: disease-free survival

ER: estrogen receptor

HT: hormone treatment

ICR: immunological constant of rejection

MCA: multiple correspondence analysis

SLFN11: Schlafen-11

TNBC: triple-negative breast cancer

INTRODUCTION

Breast cancer (BC) is the second most common cancer in the world and, by far, the most frequent neoplasm among women(1).

BC is a clinically and molecularly heterogeneous disease and genomic microarray analyses have corroborated the presence of at least four distinct intrinsic molecular subtypes: luminal A, luminal B, basal-like, and HER2 enriched subsets(2,3). These subtypes display varying degrees of sensitivity to treatment and highlight the molecular heterogeneity of BC(4).

We and an independent group(5) recently discovered the role of a putative DNA/RNA helicase, Schlafen-11 (SLFN11), for its causal association with sensitivity to DNA damaging agents (DDA), such as platinum salts, topoisomerase I and II inhibitors, and other alkylators in the NCI-60 panel of cancer cell lines(6).

SLFN11 belongs to the Schlafen protein family, which has been implicated in the regulation of important mammalian biological functions, such as control of cell proliferation(7), induction of immune responses(8), and regulation of viral replication(9).

Schlafen genes were originally identified during screening for growth regulatory genes, and they are differentially expressed during lymphocyte development(10-13). Later, SLFN11 was described as an early interferon response gene, in association with HIV infection(9). Furthermore, Murai *et al.* described molecular mechanisms detailing how SLFN11 is a dominant determinant of sensitivity to DNA-targeted therapies(14). In particular, SLFN11 inhibits checkpoint maintenance and homologous recombination by removing Replication Protein A from single-stranded DNA(15). Tang *et al.* demonstrated that the use of histone deacetylase inhibitors can be used to sensitize SLFN11-inactivated cancers to DDA(16). Recently, the importance of SLFN11 as a predictor of sensitivity to DDA has been proven in Ewing's sarcomas, ovarian cancer and colorectal cancer(17-21). SLFN11 has also been confirmed as a predictive biomarker of PARP inhibitor sensitivity in

small-cell lung cancer(22).

The aims of our study were to investigate the transcriptional landscape of SLFN11 expression in invasive BC and to identify clinical and pathological parameters that could help explain SLFN11 modulation in BC. In addition, we set up to determine whether SLFN11 expression could be associated with prognosis or response to treatment in this neoplasm.

MATERIALS AND METHODS

Datasets retrieval, pre-processing and data normalization

Thirty-five gene expression datasets of expression profiles from 7 737 tumors were retrieved from public databases or authors' websites [32 sets previously described in (23) and another three: PNC, METABRIC and TCGA(24-26). Immune phenotypes for TCGA BC cases and leucocyte infiltration were obtained as described in Hendrickx W *et al.*(27).

To ensure comparability of expression values across multiple data sets and microarray platforms (Agilent, Affymetrix or Illumina), we performed 0.95 quantile normalization (using the R/Bioconductor package genefu(28)).

SLFN 11 expression analysis and gene signature enrichment

Whole transcriptome correlation of SLFN11 was performed using Spearman's rank correlation. We selected the top 5^{th} percentile of transcripts that better correlated with SLFN11 expression. Functional annotation of correlators was further performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) v6.7(29) in order to identify significantly enriched pathways (false discovery rate (FDR) < 0.05), particularly Gene Ontology (GO) terms (The Gene Ontology Consortium). DAVID identifies GO categories to which genes belong, determining the statistical significance of non-random representation. To provide an independent assessment of enrichment analysis, we classified patients in molecular subtypes, extracting relative genomic

signatures from the *genefu* package(28). Patients labeled as "normal" PAM50 phenotype were removed, upon concerns of low cancer cellularity and possible ensuing contamination by normal breast tissue(30). The most significant gene signatures were extracted using a feature selection machine learning approach, called LASSO regression (*glmnet* package)

Multiple correspondence analysis

We investigated the modulation of SLNF11 in breast cancer through the study of the mutual distribution of clinical and pathological categorical data. First, we removed T1a samples, due to their small relative number and size, Tx and Nx tumor patients and all those patients with unknown age information, estrogen receptor or HER2 status. For this analysis, SLFN11 expression was subdivided in tertiles of expression (low, intermediate, and high). Exploratory assessment and inter-dependencies relations of data, combined with the extracted gene signatures, were accomplished by multiple correspondence analysis with the *FactoMineR* package.

Survival Analysis and time dependency correlation

Survival analyses were performed in order to determine the association of SLFN11 with prognosis in BC. We defined, by univariable statistical analysis, the association between disease-free survival (DFS) and SLFN11 expression ("low" if in the lower two tertiles and "high" if in the top tertile). The DFS curves were generated using Kaplan-Meier estimators (*survcomp* package) and p-values were obtained with the log-rank test. For what concerns the analysis of more than one covariates, we employed a stepwise backward-forward Cox proportional hazards regression model. The Akaike Information Criterion allowed the estimation of the best set of clinical and pathological variables described above (*MASS* package).

To explore time-dependency of SLFN11 modulation, we tested the proportional hazards assumption for a Cox regression model as described previously(31). We tested a two-sided hypothesis, rejecting the null ones with a p-value < 0.05 and applied multiple corrections of resulting p-values

using the Benjamini-Hochberg method.

RESULTS

SLFN11 expression correlates with BC-immune related transcripts

To investigate the transcriptional landscape of SLFN11 in BC, we conducted a gene expression microarray meta-analysis of 7 737 cases from 35 publicly available datasets.

Of 7 737 cases, we assessed 5 061 patients with SLFN11 expression values. Then, we performed a whole-transcriptome correlation analysis with SLFN11 and identified 537 genes in the top 5th percentile of correlation. The list of these 537 transcripts was analyzed for gene ontology (GO) enrichment. Strikingly, immune function processes represented most of the GO terms resulting from such analysis. The overrepresented terms in our sample set are listed in Table 1.

In agreement with such finding, we observed a strong positive association between well-established markers of tumor lymphocytic infiltration with SLFN11 expression such as CD3 and CD8 (Spearman's $\rho = 0.527$ – FDR < 0.0001 with the expression of CD3, and $\rho = 0.514$ with the expression of CD8 – FDR < 0.0001, see Figure 1).

Overall, this data purports an association of SLFN11 with immune modulation in BC.

Term	Count	Percent	Fold-en.	FDR
Immune response	117	23.2	5.62	5.94E-53
Positive regulation of immune system process	55	10.9	7.66	3.36 E-29
Cell activation	56	11.1	6.47	8.42 E-26

Leukocyte activation	52	10.3	7.12	8.74 E-26
Regulation of cell activation	44	8.7	8.34	3.08 E-24
Regulation of lymphocyte activation	41	8.1	9.19	4.14 E-24
Lymphocyte activation	45	8.9	7.50	8.28 E-23
Regulation of leukocyte activation	41	8.1	8.19	4.94 E-22
Regulation of T cell activation	35	6.9	9.92	2.69 E-21
Positive regulation of cell activation	34	6.7	10.16	5.84 E-21

Table 1: Top Gene Ontology (GO) terms associated with SLFN11 expression in BC

Fold-en = fold enrichment; FDR = false discovery rate

FIGURE 1

SLFN11 expression correlates with BC-immune gene signatures

Next, to validate our observations from an independent perspective, we inferred gene expression signatures from 4 740 patients after removing the "normal-like" intrinsic phenotype cases (in light of their low cellularity) and exploited LASSO penalized regression to extract the most relevant signatures associated with SLFN11 expression. In harmony with our previous observations, we observed an independent, strict association with immune-related signatures, in particular with two publicly described signatures '*Immune2*' (32) ($\rho = 0.508$, FDR < 0.0001) and '*Stroma1*' (32) ($\rho = 0.377$, FDR < 0.0001, see Figure 2).

FIGURE 2

High expression of SLNF11 is linked with aggressive BC

To better understand the role of SLFN11 in BC modulation, we performed multiple correspondence analysis (MCA) including clinical and pathological parameters, as well as SLFN11 levels ranked by tertiles of expression.

2 581 patients from 7 datasets, presenting with all clinical and pathological features including ER and progesterone receptor immunohistochemistry, HER2 status, grade, T, N, intrinsic subtype, and STAT1 signature as a proxy for immune activation(33) were considered for such analysis.

MCA highlighted two clearly separated patient clusters. The *"SLFN11-hot"* cluster is defined by high SLFN11 expression, ER-negative status, high histological grade, basal-like phenotype, immune activation, and younger age at diagnosis (<50 years old).

The *"SLFN11-cold"* cluster is characterized by low/intermediate SLFN11 expression, ER-positive status, lack of HER2 amplification, older age at diagnosis (>50 years old), and low/intermediate STAT1 expression (see Figure 3).

In summary, high SLFN11 expression correlates with aggressive tumors with signs of immune activation (basal-like phenotype, higher histological grade, younger age), whereas lower SLFN11 expression can be observed in luminal, less aggressive neoplasms characterized by low immune activation.

FIGURE 3

SLFN11 overexpression is independently associated with better prognosis

To evaluate whether SLFN11 expression could be associated with prognosis or response to treatment in BC, we evaluated 2 093 patients from 3 different datasets with complete information concerning DFS and type of treatment.

By univariable analysis, SLFN11 was not associated with prognosis (HR = 1.09 for SLFN11-high vs. low expression, 95% CI = 0.88-1.36, p-value = 0.37)

On the other hand, when taking into account clinical and pathological parameters as well as type of treatment and intrinsic subtypes, SLFN11 high expression was independently associated with better prognosis (HR = 0.61, 95% CI = 0.41-0.91, p-value = 0.0153). Moreover, we could define an interaction between SLFN11 expression and hormone treatment (HT), with high-SLFN11 patients undergoing HT being characterized by worse outcome (HR: 1.81, 95% CI = 1.11-2.96, p-value for interaction = 0.0175, Figure 4, panel A).

To better understand this not obvious observation, we investigated SLFN11 expression and HT in relation with possible time dependencies violating the Cox proportional hazards assumption. Indeed, in our analysis high SLFN11 levels subtended a worse prognosis in the first two years after diagnosis only in patients undergoing HT (Figure 4, panel B).

FIGURE 4

SLFN11 is independent from BC immune activation status in prognosis prediction

Finally, we derived leucocyte infiltration and immune phenotypes in the most extensively analyzed set available to us, TCGA, as previously described(27). We could indeed confirm that SLFN11 expression is associated with leucocyte infiltration (Spearman's $\rho = 0.61$, p-value < 0.0001, see Supplementary Figure S1). However, in a survival model taking into account the interaction of SLFN11 and the recently described BC "low" and "high" immunological constant of rejection (ICR) phenotypes (N = 318)(27), we could not find a significant interaction in determining prognosis between the two variables. Surprisingly, however, our model suggested that high SLFN11/high ICR cases may have a short-term worse prognosis than other cases (adjusted HR = 2.68, 95%CI = 0.28-25.56, p-value = 0.1483, with a p-value for violating the proportional hazards

assumption = 0.1114).

DISCUSSION

In the present article, we investigated for the first time how SLFN11 is modulated in BC, analyzing more of 7 000 BC cases available from 35 public datasets. Our findings demonstrated a strong correlation of SLNF11 expression with immune system transcriptomic markers, in particular with transcripts involved in immune system processes such as "*prolymphocyte activation*", "*immune response*" and "*T cell activation*". Our findings document the relationship between SLFN11 and immunity in BC, initially suggested by previous works in other settings(9,34,35). In analogy with our findings, Stewart *et al.* recently published that SLFN11 high expression in small cell lung cancer is positively correlated with immune regulatory pathways, particularly with Type 1 interferon pathway genes(34). Therefore, SLFN11 appears to have a significant role not only in innate immunity processes such as defense response to virus(9) or DNA damage repair(18), but also in adaptive immune response to cancer.

SLFN11, in addition to its known expression by cancer cells(18) could indeed be expressed by immune cells during anti-tumoral response, potentially behaving as a marker of T-cell infiltration in BC as well as in other tumor types. The consistent association of SLNF11 with immunity is exemplified by its strong correlation with tumor infiltrating lymphocyte markers(CD3 and CD8 in our analyses).

Of note, we identified a strong independent correlation of SLFN11 with two immune gene signatures, namely *stroma1* and *immune2*. In the last few years, several prognostic and/or predictive gene expression signatures have been published in BC(36-38). Desmedt *et al.*, in their comprehensive meta-analysis, showed as several prognostic gene signatures differ in prognostic abilities according to the BC subtype and as only immune response modules seem to predict

prognosis in ER-negative/HER2 negative BC patients(33). On the other hand, we previously pointed out the prognostic and predictive value of immune gene signatures in primary TNBC underlining the activation of Th1/effector immune response(36). Our findings show both high expression of SFLN11 in a subgroup of patients with TNBC-like features and a strong correlation with immune signatures, in particular *immune2*, supporting an involvement of SLFN11 during the effector immune response in BC. In parallel, stroma signatures have also been developed in BC in order to predict clinical outcome and treatment response(39-41). Particularly, Finak *et al.* developed a 26 gene stroma-derived prognostic predictor in which a good-outcome cluster overexpresses a distinct set of immune-related genes, including T cell and NK cell markers indicative of a Th1–type immune response (GZMA, CD52, CD247, CD8A)(42). Winslow *et al.* showed that a specific immune gene signature (C1Q), represented by genes such as DZMH, GZMA, GZMK, CD3D, CD3G, CD247, CD8A, coding for proteins involved in cytotoxic immune response in TNBC, is associated with low risk of recurrence. Finally, their results support that the molecular profile of a Th-1/immune response ($CD4^+$ T cells) is an important prognostic marker in BC(40) as also hypothesized by Gu-Trantien *et al.* in her work(43). In good agreement with such independent observations, in our study SFLN11 is highly associated with stromal signatures, in particular *stroma1,* and expression of T-cell markers, supporting the idea of a role of SLFN11 in Th-1/effector immune response in BC.

Through our unbiased analysis of SLFN11 expression in relation with clinico-pathological BC variables, we discovered two distinct BC patient subgroups. In the "*SLFN11-hot"* cluster, we observed a high expression of the signature of STAT1, a key mediator of type I and type II interferon response. Among its many functions, STAT1 promotes Th1 immune response and TCD8+ cell recruitment(44). This type of immune activation is predominant in TNBC, a subgroup of BC that is considered highly immunogenic. TNBC typically presents a worse prognosis than other BC subtypes, with – however – a very heterogeneous response to current systemic

chemotherapies and absence of actionable molecular targets. To overcome this issue, current clinical trials testing a combination of immunotherapy and chemotherapy in TNBC are ongoing(45).

In our analysis, we demonstrated that SLFN11 expression is strictly related to BC-immunity, in particular in TNBC. The "*SLFN11-hot"* cluster encompasses a distinct BC subgroup with TNBC-like features, strong immune activation, better prognosis and better response to systemic treatments compared to other BC subtypes. On the other hand, the *"SLFN11-cold"* cluster might represent a different subgroup of scarcely immunogenic BC with minor response to systemic treatment. Therefore, SLFN11 as immune-related biomarker is an intriguing venue for further translational research.

In our time dependency analysis, we identified a subgroup of high-SLFN11 BC patients treated with HT presenting with worse outcome in the first two years of follow-up. This behavior shows similarities with TNBC and suggests that the phenomenon that we observed might be actually due to a subset of hormone receptor-poor patients with a biological behavior analogous to that of TNBC. This is, however, just a hypothesis since we did not have the availability of ER expression level by immunohistochemistry in the evaluated dataset for a precise quantitation of ER by standard methods. Our observation is in agreement with recent literature, since several papers confirmed the analogies between TNBC and Luminal-B BC concerning survival rates(46), response to neoadjuvant chemotherapy(47), high mutational burden, and immunogenic profile characterized by higher expression of TIL(48). Finally, Luminal-B BC are poorly responsive to HT(49), and could be stratified by immune profile analysis into different prognostic groups(50), so that in future studies on BC, we believe SLFN11 expression should be assessed together with other established parameters for prognostic and predictive purposes.

Our lack of identifying a clear association between SLFN11 levels and immune activation in BC in determining prognosis is somehow puzzling. We may speculate that SLFN11 levels in cancer cells

play an independent role in response to DDAs when considered together with immune status in BC. As a consequence, we strongly advocate for future studies to morphologically deconvolute SLFN11 expression in cancer cells and in immune infiltrate in selected BC cohorts to reach a causal understanding of the role of this protein. On the other hand, our inconclusive results in assessing the relation of SLFN11 and immune activation in BC may be due to both the relatively low number of events (N = 42) and the insufficient length of the follow-up time (median 2.5 years) of publicly available BC TCGA data. Moreover, the suggestion of a worse short-term prognosis again favors the idea of high SLFN11 being a characteristic of BC cancer with such behavior, as TNBC is. The negative prognostic effect of SLFN11 in the high ICR BC cases is puzzling, and we should be careful in overinterpreting substantially indecisive results. Our analyses have several limitations. Amongst them the heterogeneity concerning the origin of data, chip design, and clinical annotation are unavoidable. Moreover, we did not perform preclinical experiments for our findings, which are of associative nature so far - albeit suggestive -, and SLFN11 location in BC is yet to be determined, since the contribution from infiltrating lymphocytes may be determinant in this regard.

CONCLUSION

In summary, a consistent and evident pattern emerges, highlighting the strong correlation of SLFN11 with the immune system in BC, as well as its meaningful associations with clearly distinct clinico-pathological BC phenotypes and clinical outcome. Further studies will have to focus on biological, well-annotated and homogeneous specimens from clinical BC cohorts to further unravel SLFN11 role in BC.

Conflict of interest:

The authors declare no conflict of interest.

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Figure and Figure legends

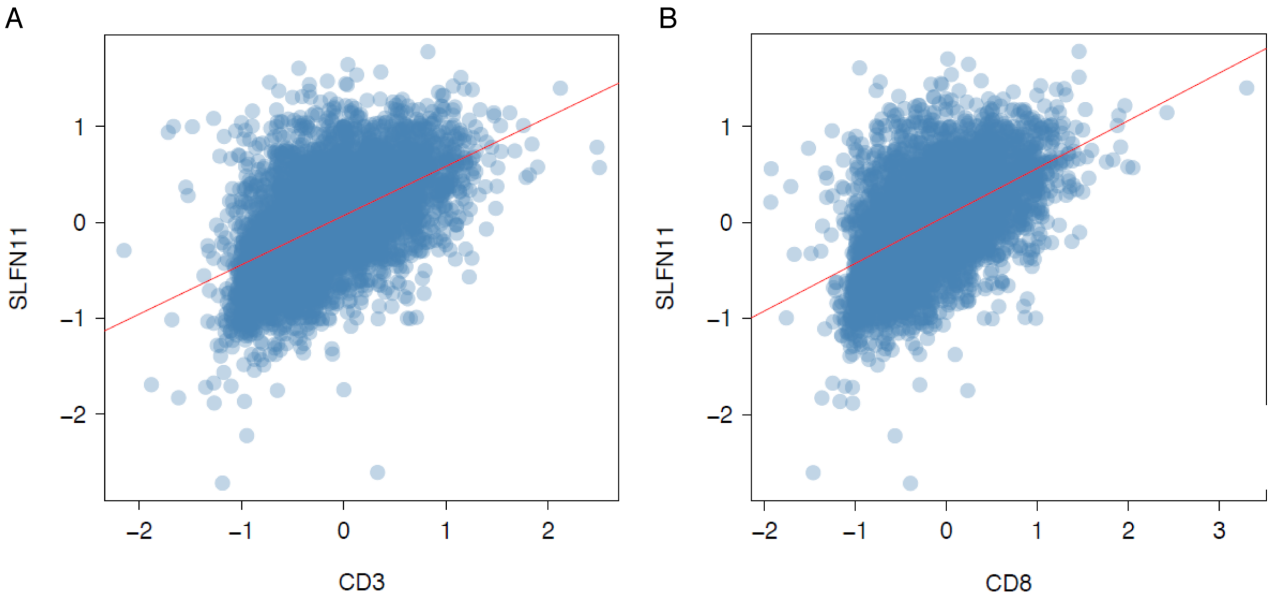

Figure 1. Panel A: Correlation between SLFN11 (*y-axis*, z-score gene expression values) and CD3 (*x-axis*, z-score gene expression values). Panel B: Correlation between SLFN11 (*y-axis*, z-score gene expression values) and CD8 (*x-axis*, z-score gene expression values).

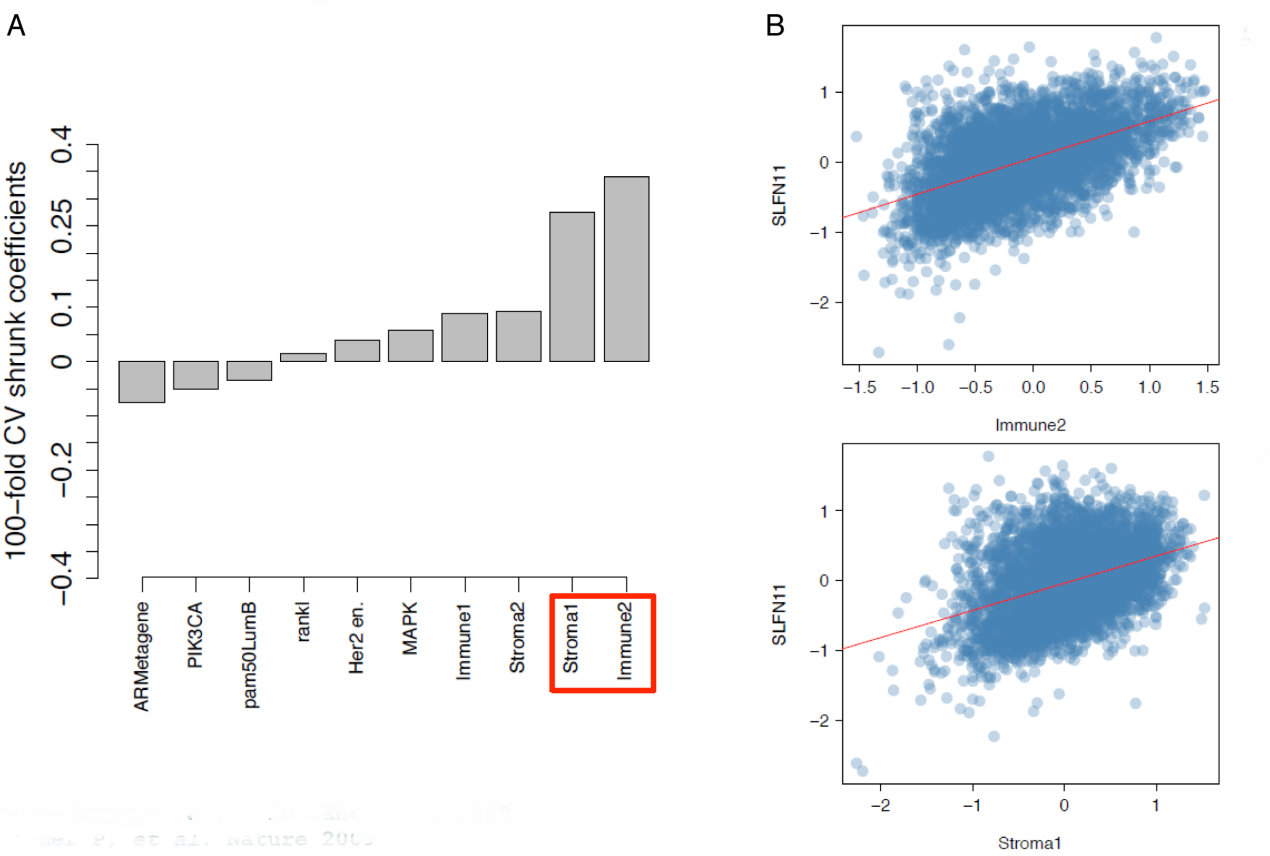

Figure 2. Panel A: bar plot shows the LASSO regression coefficient weights related to the gene signatures of interest: the highest weighted signatures are highlighted in a red contoured box. Panel B: The upper and lower scatterplots show the correlation between SLNF11 and the most relevant gene signatures resulting from previous variable selection analysis.

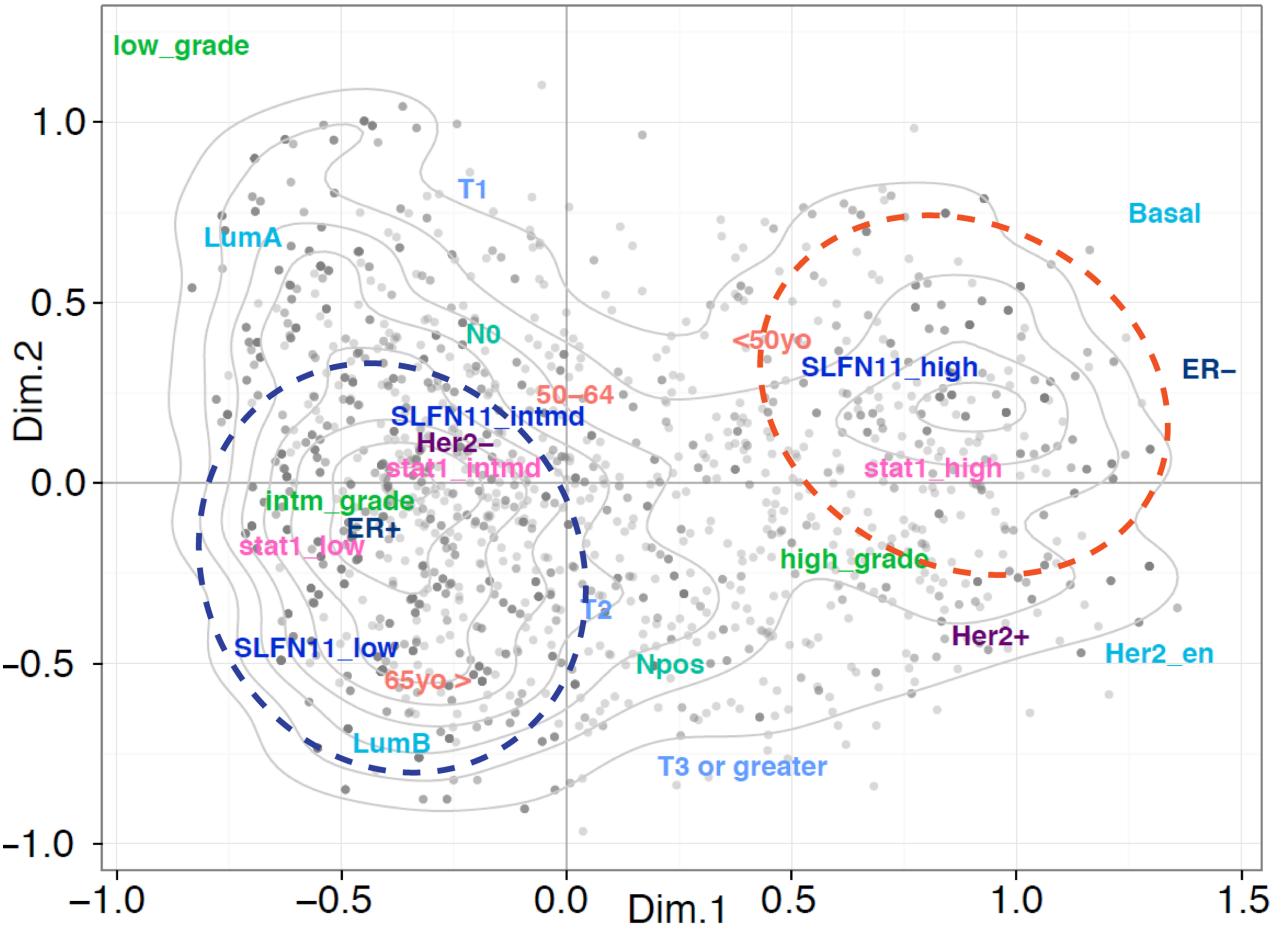

Figure 3. MCA showing the relationship patterns between clinico-pathological variables and SLFN11 expression in breast cancer.

x- and y-axes represent the first and second dimension (Dim.1 and Dim.2) of the MCA analysis performed on clinical and pathological data, as well as SLFN11 expression, divided in tertiles, from 2,581 BC patients. Patients are represented by small grey dots and categorical variables are colored. In particular, patients with high-grade tumors also show high SLFN11 expression levels (highlighted by the red dashed circle), whereas the cluster of patients with SLFN11 low and

intermediate expression tumors is also characterized by low and intermediate (Low/Intm) STAT1 expression, HER2-, ER+ cancers (steel-blue dashed circle).

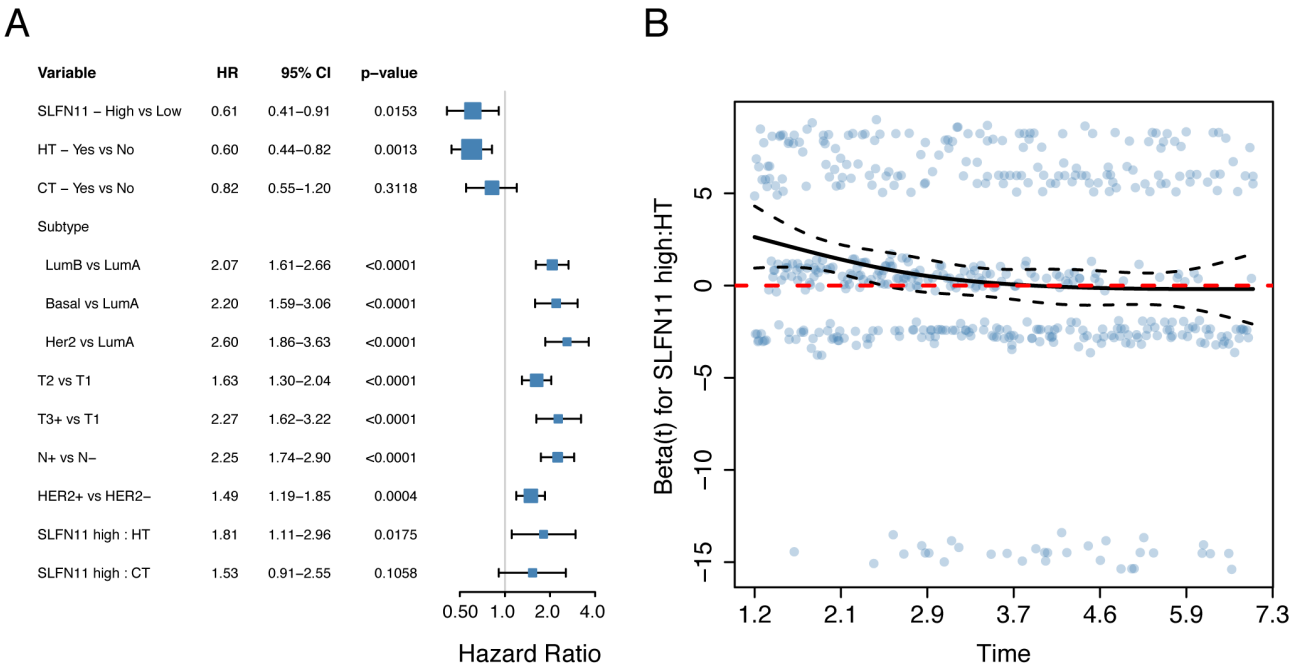

Figure 4. Panel A: Forest plot of Cox regression model for DFS in 2,093 BC patients with complete anatomopathological and clinical follow-up data. Panel B: Plot of scaled Schoenfeld residuals. Red dashed and blue dotted lines represent, respectively, the null effect (null log hazard ratio) and a ± 2-standard-error band around the fit. On the *x-axis*, time is expressed in years.

6) *Annex:*

i. *Supplementary Paper:*

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Personalized Medicine and Imaging

Clinical Cancer Research

Location of Mutation in *BRCA2* Gene and Survival in Patients with Ovarian Cancer

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Abstract

Purpose: BRCA2 plays a central role in homologous recombination by loading RAD51 on DNA breaks. The objective of this study is to determine whether the location of mutations in the RAD51-binding domain (RAD51-BD; exon 11) of *BRCA2* gene affects the clinical outcome of ovarian cancer patients.

Experimental Design: A study cohort of 353 women with ovarian cancer who underwent genetic germline testing for *BRCA1* and *BRCA2* genes was identified. Progression-free survival (PFS), platinum-free interval (PFI), and overall survival (OS) were analyzed. The Cancer Genome Atlas (TCGA) cohort of ovarian cancer ($n = 316$) was used as a validation cohort.

Results: In the study cohort, 78 patients were carriers of germline mutations of *BRCA2*. After adjustment for FIGO stage and macroscopic residual disease, *BRCA2* carriers with truncating mutations in the RAD51-BD have significantly prolonged 5-year PFS [58%; adjusted HR, 0.36; 95% confidence interval (CI), 0.20–0.64; $P = 0.001$] and prolonged PFI (29.7 vs. 15.5 months, $P = 0.011$), compared with noncarriers. *BRCA2* carriers with mutations located in other domains of the gene do not have prolonged 5-year PFS (28%, adjusted HR, 0.67; 95% CI, 0.42–1.07; $P = 0.094$) or PFI (19 vs. 15.5 months, $P = 0.146$). In the TCGA cohort, only *BRCA2* carriers harboring germline or somatic mutations in the RAD51-BD have prolonged 5-year PFS (46%; adjusted HR, 0.30; 95% CI, 0.13–0.68; $P = 0.004$) and 5-year OS (78%; adjusted HR, 0.09; 95% CI, 0.02–0.38; $P = 0.001$).

Conclusions: Among ovarian cancer patients, *BRCA2* carriers with mutations located in the RAD51-BD (exon 11) have prolonged PFS, PFI, and OS. *Clin Cancer Res; 24(2); 326–33. ©2017 AACR.*

Introduction

Germline mutations in *BRCA1* and *BRCA2* genes have been identified as predisposing to hereditary breast and ovarian cancers (1). Up to 20% of high-grade serous ovarian carcinomas (HGSOC) show germline and/or somatic mutations of *BRCA1*/*BRCA2* genes (2–5). Ovarian cancer patients with such mutations have better survival than noncarriers (2, 6, 7). Importantly, almost all the ovarian cancer patients in those studies received platinum-based chemotherapy, inducing interstrand crosslinks (8). Interstrand crosslinks formed by platinum lead to severe distortion of the DNA double helix, and consequently double-strand breaks (DSB; refs. 9, 10). Homologous recombination (HR), a major mechanism for protecting genome integrity in proliferating cells, is pivotal in repairing DSBs that arise during the processing of interstrand crosslinks.

From a biological point of view, BRCA1 and BRCA2 are both key players of DNA damage repair but have different functions (11). BRCA1 is a pleiotropic DNA damage response protein that functions in both DNA damage checkpoint activation and repair, including HR (11, 12). In contrast, the primary function of BRCA2 is HR. BRCA2 interacts directly with RAD51 and promotes its specific recruitment to DSBs sites where recombination is initiated (11). RAD51 is essential for HR. The improved survival of *BRCA1*/*BRCA2* germline mutation carriers, especially *BRCA2* carriers, compared with noncarriers (13, 14) has been linked to the role of these proteins in the HR pathway. However, there is no clear explanation of why *BRCA2* carriers fare better than *BRCA1* carriers.

BRCA2 is one of the largest proteins in the human body. The central portion of the protein contains 8 repeat sequences (called BRC repeats), which bind to RAD51, named RAD51-binding domain (RAD51-BD; refs. 11, 15). A second

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

BRCA2 plays a central role in homologous recombination by loading RAD51 on DNA double-strand breaks. Ovarian cancer patients who were carriers of *BRCA2* germline mutation and were treated with DNA damage agent platinum showed prolonged survival. We questioned whether the location of the mutation in various functional domains of BRCA2 has an impact on the clinical outcome for ovarian cancer patients. In two independent cohorts of ovarian cancer patients, we observed that only those patients whose germline or somatic mutations of *BRCA2* are located within the RAD51-binding domain of the protein have prolonged platinum-free interval and survival, compared with noncarriers. These results suggest that not all *BRCA2* carriers are highly sensitive to DNA damage agents, and the response depends on the location of the mutation in the various functional domains of the protein.

large segment of BRCA2 encompasses a DNA-binding domain (Fig. 1). Genetic and functional studies of BRCA2 revealed that mutations located in RAD51-BD impair the ability of BRCA2 to recruit RAD51, hampering HR (16, 17). In the current report, we investigated whether mutations in the RAD51-BD (exon 11) of the *BRCA2* gene impact progression-free survival (PFS), platinum-free interval (PFI), and overall survival (OS) in ovarian cancer patients.

Materials and Methods

Clinical and genetic data were collected from a study cohort of ovarian cancer patients screened for germline mutations of *BRCA1* and *BRCA2* genes. We analyzed the curated dataset of HGSOC from The Cancer Genome Atlas (TCGA) as a validation cohort.

Study cohort

Study participants were women with confirmed invasive epithelial ovarian or fallopian tube or peritoneal carcinoma, who had been tested for germline *BRCA1* or *BRCA2* pathogenic mutations through blood tests between January 1995 and December 2015 and who received platinum-based chemotherapy. Ovarian cancer patients referred to the clinical genetics Units' at Centre Leon Bérard (Lyon, France), Institut du Cancer Jean Mermoz (Lyon, France), and Hôpitaux Universitaires de Genève (Geneva, Switzerland) were included in the cohort. To increase the number of *BRCA2* carriers in the study cohort, only *BRCA2* carriers were included from Institut Curie (Paris, France). The study was conducted following ethical guidelines of the Declaration of Helsinki. The study was reviewed by the local Institutional Review Board in each hospital. Informed consent was obtained from all living patients in Geneva. All the French patients consented to the use of their data at the time of genetic analysis. Clinical and pathologic data were collected from medical records. These included patient demographics, tumor characteristics, surgical staging, macroscopic residual disease, platinum sensitivity, recurrence, and survival status. Surgical stage was classified according to the International Federation of Gynecology and Obstetrics (FIGO) at diagnosis. Information regarding residual disease following primary surgery was acquired from medical records. Pathology data, including histologic subtypes, tumor stages, and grades, were obtained from pathology reports.

Genetic analysis

BRCA1 and *BRCA2* mutations were classified as truncating according to the ENIGMA *BRCA1/2* Gene Variant Classification Criteria (http://www.enigmaconsortium.org/). Women with variants of uncertain significance were considered noncarriers. Blood samples for germline DNA testing were obtained when the patients were referred to clinical genetics Unit. All participants were screened for *BRCA1* and *BRCA2* mutations. The *BRCA2* gene comprises 27 exons and encodes a 3418 amino-acid (AA) protein (Fig. 1). RAD51-BD corresponds to the region covering AA 1003-2082 of BRCA2 (exon 11). DNA-BD corresponds to AA 2481-3186 (http://www.ncbi.nlm.nih.gov/protein/NP_000050.2). The BRCA2 protein has a second binding site to RAD51 located in the carboxy-terminus (named RAD51-binding site or TR2), which we excluded from the analysis because it is dispensable for HR (11, 12).

TCGA cohort

We obtained the TCGA database of 316 HGSOC patients. Detailed patient information, including age at diagnosis, tumor stage and grade, and surgical outcome, has been described previously (13). Whole-exome sequencing of germline and somatic DNA was performed in all cases (2).

Outcome measures

The primary endpoint was PFS. Secondary endpoints were PFI and OS. Date of first relapse was defined as the first instance of disease progression based on CT imaging by RECIST or clinical progression. PFS was defined as the interval between histologic diagnosis and first relapse, death, or the last follow-up (censored). OS was defined as the interval between histologic diagnosis and the date of death from any cause or last follow-up (censored). PFI was defined as the interval between the time of completion of platinum-based chemotherapy and the date of first relapse or death. Platinum-sensitive patients were defined as those having PFI >6 months.

Statistical analysis

Patient characteristics were compared using Pearson χ^2 or Fisher exact test for frequencies, and Kruskal–Wallis test for age distribution. Characteristics were compared pair by pair (*BRCA2* carriers' vs. noncarriers). The survival functions of the different subgroups were estimated using the Kaplan–Meier method and compared using a log-rank test. The relative hazards for each mutation group were estimated with a Cox proportional hazards model adjusted for tumor stage and macroscopic residual disease. The categorical covariates included in the model were FIGO stage (stage I–II vs. III–IV) and macroscopic residual disease (absent vs. present). $P \leq 0.05$ was considered statistically significant; all tests were two-sided. Statistical analysis was carried out using R software.

Results

Characteristics of the study cohort

Of the 353 women included in the cohort, 87 were excluded from the analysis: 74 women were found to carry *BRCA1* mutation

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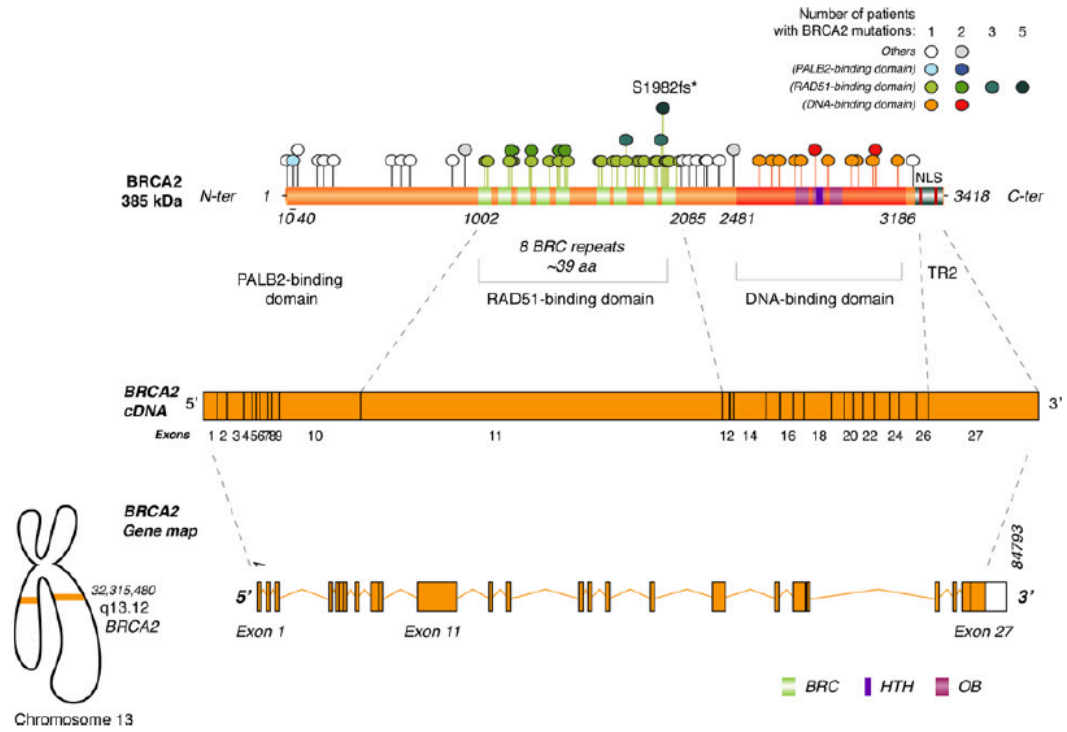

Figure 1.

Germline mutations of *BRCA2* gene in the study cohort of ovarian cancer patients. The human *BRCA2* gene is located on the long arm of chromosome 13 (13q12.3) and is composed of 27 exons that encode for a protein of 3,418 amino acids. The N-terminal domain of BRCA2 is involved in interaction with PALB2. BRCA2 contains 8 BRC repeats located in the central portion of the protein; they are primarily involved in binding to monomeric RAD51 (RAD51 binding domain: RAD51-BD). The BRCA2 DNA-binding domain (DNA-BD) promotes BRCA2 binding to single-stranded DNA (ssDNA) and poly(ADP-ribose). The C-terminus of BRCA2 contains the TR2 domain, which interacts with RAD51 nucleofilaments.

(they will be analyzed in another study), 2 patients harbored mutations in both *BRCA1* and *BRCA2* genes, 9 patients did not receive adjuvant chemotherapy (5 noncarriers, 1 *BRCA1*, and 3 *BRCA2* carriers), and follow-up data were not available for 2 noncarriers (Supplementary Fig. S1). In total, 266 women (152 from Centre Leon Bérard, 40 from Hôpitaux Universitaires de Genève, 26 from Institut du Cancer Jean Mermoz, and 48 from Institut Curie) were analyzed for outcome: 188 were tested negative for germline mutations in *BRCA1* and *BRCA2* genes (hereafter "noncarriers") and 78 were found to carry *BRCA2* mutations (hereafter "*BRCA2* carriers"). All mutations were germline. Patient demographics and clinical and treatment characteristics are summarized in Table 1.

The majority of patients had serous carcinomas and advanced stages (III/IV; 88%). Two thirds (67%) of the patients had no macroscopic residual disease and 85% were platinum sensitive. All patients received a platinum agent, and 95% of the patients received a combination with taxane.

There was no difference between the two groups regarding age of diagnosis ($P = 0.814$), histologic subtype ($P = 0.451$), FIGO staging ($P = 0.655$), and the presence of macroscopic residual disease ($P = 0.15$). Platinum sensitivity rates were not statistically different in the two groups of patients: 85% of noncarriers and 86% of *BRCA2* carriers were sensitive ($P = 0.722$). However, *BRCA2* carriers had longer PFI ($P = 0.008$).

BRCA mutation and survival in the study cohort

Median follow-up of the cohort was 4 years. After adjustment for major prognostic factors (stage and macroscopic residual disease), PFS at 5 years was significantly higher in *BRCA2* compared with noncarriers ($P < 0.001$; Fig. 2A; Supplementary Table S1). Regarding OS, *BRCA2* carriers had significantly higher 5-year OS compared with noncarriers ($P < 0.001$; Supplementary Table S2).

Location of mutations in BRCA2 and clinical outcome in the study cohort

Among the 78 *BRCA2* carriers of the study cohort, 42 had mutations located within the RAD51-BD (Fig. 1; Supplementary Table S3), including 5 carriers of the Ashkenazi founder mutation c.5946del/p.Ser1982Argfs*22. They had no macroscopic residual disease ($P = 0.009$) more frequently and had significantly prolonged PFI ($P = 0.011$), compared with noncarriers. There was no significant difference between the 36 other *BRCA2* carriers and noncarriers, regarding any of the clinical, pathology, and treatment characteristics (Table 1).

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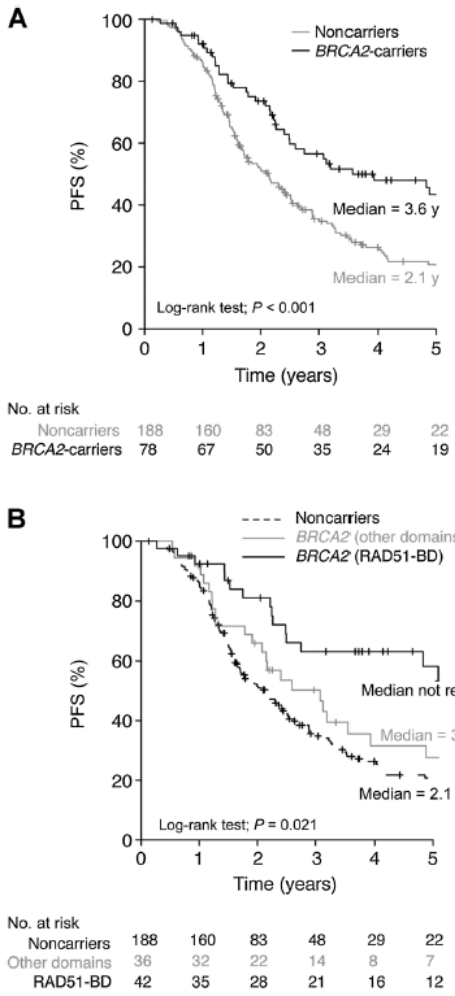

Figure 2.

BRCA2 genotype correlates with PFS in the study cohort. **A,** PFS for *BRCA2* carriers and noncarriers. **B,** PFS for *BRCA2* carriers having mutations located in RAD51-BD (exon 11), other *BRCA2* carriers, and noncarriers.

RAD51 and BRC repeats could impair the ability of BRCA2 to recruit RAD51 to DNA breaks, hampering HR (Supplementary Fig. S2; refs. 16, 17) and impacting patients' survival.

Of course, this study has several limitations. First, the cohort is derived from a retrospective study that only included patients screened for germline mutations. The criteria for genetic analysis of *BRCA1*/*BRCA2* genes have evolved over time. Systematic screening for all ovarian cancer patients has recently been implemented. Thus, our cohort study included patients selected for their young age at diagnosis and/or significant family history of cancer and does not reflect the general population of ovarian cancer patients. Second, our noncarrier group probably included some patients with somatic mutations of *BRCA1*, *BRCA2*, and other HR genes (2). The enrichment in *BRCA2* carriers, who were mainly recruited at Institut Curie could also be considered as a limitation. On the other hand, compared with the literature, our study cohort is representative of *BRCA2* carriers in terms of clinical characteristics (age of diagnosis, histologic subtype, stage, etc.) and clinical behavior with *BRCA2* carriers showing significantly prolonged survival compared with noncarriers (4, 13).

Two thirds of the patients included in the study cohort had no macroscopic residual disease, explaining high survival rates, as described previously (19). The low number of events in the *BRCA2* carriers group allowed subgroup investigation only for PFS, not OS. OS data for *BRCA2* patients will require longer follow-up before subgroup analysis. It should be noted that PFS has been shown to be a surrogate marker for OS in *BRCA* carriers in the TCGA cohort (13) and the GOG clinical trials 218 and 262 (4). Moreover, we observed in the TCGA cohort that OS was dramatically prolonged in these patients. One major difference between our study cohort and the TCGA cohort is the percentage of patients with no macroscopic residual disease, which was lower in the TCGA cohort (66% vs. 20%, respectively).

Our study and the TCGA cohort showed that only mutations in the RAD51-BD of *BRCA2* gene lead to prolonged PFS, PFI, and OS in ovarian cancer patients, compared with noncarriers. As BRCA2 is a very large protein, it is possible that mutations in other domains of the protein could also impact clinical outcome of ovarian cancer patients. In our study cohort, we did not observe that mutations located in the DNA-BD impact PFS.

A very large proportion of pathogenic *BRCA2* mutations are truncating deletions, meaning that cells containing early mutations (exons 1–10) are likely to produce no BRCA2 protein. In an exploratory analysis, we compared *BRCA2* carriers with mutations located in either exons 1–10 or exons 12–27, and we did not observe a significant difference in the outcome of both groups. Our analysis is limited by the number of cases in each subgroup

Table 2. Multivariate model of PFS in the study cohort of patients with ovarian cancer according to location of mutations in *BRCA2* gene

Variable	3-year PFS rate (%) (95%CI)	5-year PFS rate (%) (95% CI)	HR (95% CI)	*P*
BRCA				
Noncarriers	36 (29–44)	21 (15–29)	1	
BRCA2- carriers other domains	50 (36–70)	28 (15–50)	0.67 (0.42–1.07)	0.094
BRCA2- carriers RAD51-BD	63 (49–81)	58 (43–79)	0.36 (0.20–0.64)	0.001
FIGO stage				
1–2	73 (59–91)	65 (49–86)	1	
3–4	37 (31–45)	22 (16–29)	2.68 (1.34–5.36)	0.005
Macroscopic residual disease				
Absent	55 (47–63)	38 (31–48)	1	
Present	15 (83–25)	6 (23–15)	3.20 (2.29–4.47)	<0.001

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Translational Relevance

BRCA2 plays a central role in homologous recombination by loading RAD51 on DNA double-strand breaks. Ovarian cancer patients who were carriers of *BRCA2* germline mutation and were treated with DNA damage agent platinum showed prolonged survival. We questioned whether the location of the mutation in various functional domains of BRCA2 has an impact on the clinical outcome for ovarian cancer patients. In two independent cohorts of ovarian cancer patients, we observed that only those patients whose germline or somatic mutations of *BRCA2* are located within the RAD51-binding domain of the protein have prolonged platinum-free interval and survival, compared with noncarriers. These results suggest that not all *BRCA2* carriers are highly sensitive to DNA damage agents, and the response depends on the location of the mutation in the various functional domains of the protein.

large segment of BRCA2 encompasses a DNA-binding domain (Fig. 1). Genetic and functional studies of BRCA2 revealed that mutations located in RAD51-BD impair the ability of BRCA2 to recruit RAD51, hampering HR (16, 17). In the current report, we investigated whether mutations in the RAD51-BD (exon 11) of the *BRCA2* gene impact progression-free survival (PFS), platinum-free interval (PFI), and overall survival (OS) in ovarian cancer patients.

Materials and Methods

Clinical and genetic data were collected from a study cohort of ovarian cancer patients screened for germline mutations of *BRCA1* and *BRCA2* genes. We analyzed the curated dataset of HGSOC from The Cancer Genome Atlas (TCGA) as a validation cohort.

Study cohort

Study participants were women with confirmed invasive epithelial ovarian or fallopian tube or peritoneal carcinoma, who had been tested for germline *BRCA1* or *BRCA2* pathogenic mutations through blood tests between January 1995 and December 2015 and who received platinum-based chemotherapy. Ovarian cancer patients referred to the clinical genetics Units' at Centre Leon Bérard (Lyon, France), Institut du Cancer Jean Mermoz (Lyon, France), and Hôpitaux Universitaires de Genève (Geneva, Switzerland) were included in the cohort. To increase the number of *BRCA2* carriers in the study cohort, only *BRCA2* carriers were included from Institut Curie (Paris, France). The study was conducted following ethical guidelines of the Declaration of Helsinki. The study was reviewed by the local Institutional Review Board in each hospital. Informed consent was obtained from all living patients in Geneva. All the French patients consented to the use of their data at the time of genetic analysis. Clinical and pathologic data were collected from medical records. These included patient demographics, tumor characteristics, surgical staging, macroscopic residual disease, platinum sensitivity, recurrence, and survival status. Surgical stage was classified according to the International Federation of Gynecology and Obstetrics (FIGO) at diagnosis. Information regarding residual disease following primary surgery was acquired from medical records. Pathology data, including histologic subtypes, tumor stages, and grades, were obtained from pathology reports.

Genetic analysis

BRCA1 and *BRCA2* mutations were classified as truncating according to the ENIGMA *BRCA1/2* Gene Variant Classification Criteria (http://www.enigmaconsortium.org/). Women with variants of uncertain significance were considered noncarriers. Blood samples for germline DNA testing were obtained when the patients were referred to clinical genetics Unit. All participants were screened for *BRCA1* and *BRCA2* mutations. The *BRCA2* gene comprises 27 exons and encodes a 3418 amino-acid (AA) protein (Fig. 1). RAD51-BD corresponds to the region covering AA 1003-2082 of BRCA2 (exon 11). DNA-BD corresponds to AA 2481-3186 (http://www.ncbi.nlm.nih.gov/protein/NP_000050.2). The BRCA2 protein has a second binding site to RAD51 located in the carboxy-terminus (named RAD51-binding site or TR2), which we excluded from the analysis because it is dispensable for HR (11, 12).

TCGA cohort

We obtained the TCGA database of 316 HGSOC patients. Detailed patient information, including age at diagnosis, tumor stage and grade, and surgical outcome, has been described previously (13). Whole-exome sequencing of germline and somatic DNA was performed in all cases (2).

Outcome measures

The primary endpoint was PFS. Secondary endpoints were PFI and OS. Date of first relapse was defined as the first instance of disease progression based on CT imaging by RECIST or clinical progression. PFS was defined as the interval between histologic diagnosis and first relapse, death, or the last follow-up (censored). OS was defined as the interval between histologic diagnosis and the date of death from any cause or last follow-up (censored). PFI was defined as the interval between the time of completion of platinum-based chemotherapy and the date of first relapse or death. Platinum-sensitive patients were defined as those having PFI >6 months.

Statistical analysis

Patient characteristics were compared using Pearson χ^2 or Fisher exact test for frequencies, and Kruskal–Wallis test for age distribution. Characteristics were compared pair by pair (*BRCA2* carriers' vs. noncarriers). The survival functions of the different subgroups were estimated using the Kaplan–Meier method and compared using a log-rank test. The relative hazards for each mutation group were estimated with a Cox proportional hazards model adjusted for tumor stage and macroscopic residual disease. The categorical covariates included in the model were FIGO stage (stage I–II vs. III–IV) and macroscopic residual disease (absent vs. present). $P \leq 0.05$ was considered statistically significant; all tests were two-sided. Statistical analysis was carried out using R software.

Results

Characteristics of the study cohort

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investigating the PFS and OS of *BRCA2* carriers treated with PARP inhibitors based on their genotype.

In conclusion, our data suggest that PFI and survival in *BRCA2* carriers are prolonged specifically when the mutations occur in the RAD-51 BD (exon 11) of the gene. These results confirmed the new paradigm of personalized therapy in *BRCA* carriers.

Disclosure of Potential Conflicts of Interest

S.I. Labidi-Galy, M. Rodrigues, and A. Bodmer are consultant/advisory board members for AstraZeneca. O. Tredan is a consultant/advisory board member for AstraZeneca, Lilly, Novartis, Pfizer, and Roche. M-H. Stern has ownership interests (including patents) at Myriad Genetics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S.I. Labidi-Galy, T. Olivier, O. Tredan, P.O. Chappuis, I. Ray-Coquard

Development of methodology: S.I. Labidi-Galy, T. Olivier, P.O. Chappuis, A. Buisson, I. Ray-Coquard

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.I. Labidi-Galy, T. Olivier, M. Rodrigues, D. Ferraioli, O. Derbel, A. Bodmer, N. Chopin, O. Tredan, P.-E. Heudel, S. Stuckelberger, P. Meeus, V. Viassolo, A. Ayme, P.O. Chappuis, C. Houdayer, D. Stoppa-Lyonnet, L. Golmard, V. Bonadona, I. Ray-Coquard

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.I. Labidi-Galy, T. Olivier, M. Rodrigues, P. Petignat, P. Meraldi, P.O. Chappuis, M.-H. Stern, A. Buisson, L. Golmard, I. Ray-Coquard

Writing, review, and/or revision of the manuscript: S.I. Labidi-Galy, T. Olivier, M. Rodrigues, D. Ferraioli, O. Derbel, A. Bodmer, P. Petignat, B. Rak, O. Tredan, P.-E. Heudel, S. Stuckelberger, V. Viassolo, A. Ayme, P.O. Chappuis, C. Houdayer, D. Stoppa-Lyonnet, A. Buisson, L. Golmard, V. Bonadona, I. Ray-Coquard

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.I. Labidi-Galy, T. Olivier, M. Rodrigues, N. Chopin, C. Houdayer, L. Golmard

Study supervision: S.I. Labidi-Galy, T. Olivier, I. Ray-Coquard

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The results shown here are based in part on data generated by TCGA Research Network: http://cancergenome.nih.gov.

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AACR American Association for Cancer Research

Clinical Cancer Research

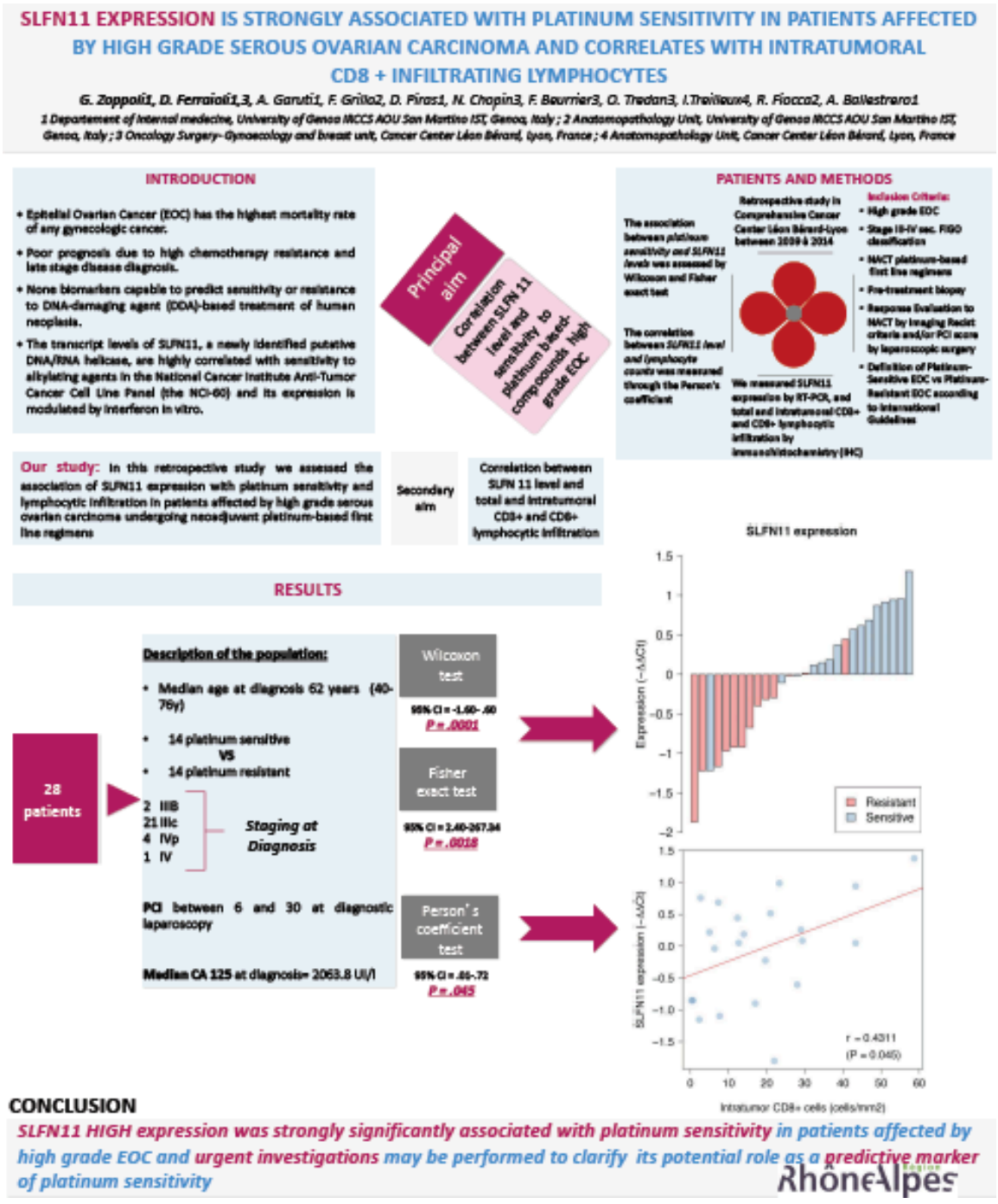
Location of Mutation in *BRCA2* Gene and Survival in Patients with Ovarian Cancer

S. Intidhar Labidi-Galy, Timothée Olivier, Manuel Rodrigues, et al.

Clin Cancer Res 2018;24:326-333. Published OnlineFirst October 30, 2017.

ii. ***Abstract and Poster Session ESGO 2015:***

Slfn11 expression is strongly associated with platinum sensitivity in patients affected by high grade serous ovarian carcinoma and correlates with intratumoral cd8 + infiltrating lymphocytes.



This abstract will soon become a manuscript to submit an immunology journal. We are waiting for the IHC from Astra Zeneca, Cambridge UK

iii. ***Submission in Journal of Pathology***

Title: ***The emergence of SLFN11 as a biomarker for DNA damaging agents and the need for antibody standardization"***

Authors: ***Domenico Ferraioli***[1]***,*** Alberto Ballestrero[1,2], Gabriele Zoppoli[1,2]

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1) Schlafen 11: General description, discovery and development

In the field of cancer therapeutics, the concept of precision medicine is based on the premise that treatment choices tailored to individual patients using personalized cancer genomic data may markedly improve outcomes.[1]

However, few anticancer drugs are currently prescribed based upon predictive markers. Precision in the clinical application of DNA- targeting drugs remains a substantially unmet need. Over the last few years, the expression of Schlafen 11 (SFLN11), a putative DNA/RNA helicase belonging to the Schlafen gene family has emerged as a promising predictive biomarker of sensitivity to DNA-targeting agents. Schlafen genes are highly conserved in mouse and humans, and they first identified in 1998 by a research group from the University of California led by Stephen Hedrick. These genes were named “Schlafen" from the German word meaning, "to sleep", in relation to the capacity of Schlafen to cause G0/G1 cell cycle arrest and to induce grown inhibition.[2]

SLFN11 has been implicated in the control of cell proliferation in response to DNA damaging agents (DDA)[3, 4] and PARP Inhibitors[5], induction of immune response[6, 7] and regulation of viral replication.[8-10]

In 2012, two independent research groups described an unusually tight correlation between the levels of SLFN11 expression and the sensitivity of response to DDA such as Topoisomerase inhibitors 1 and 2, Alkylating agents (Cisplatin) in the NCI-60 cell line panel[3] and in Ewing's sarcoma (EWS) cell lines from the Cancer Cell Line Encyclopaedia (CCLE)[4], respectively. In addition, subsequent in vitro and in vivo studies confirmed this causal relationship between SLFN11 intracellular levels and sensitivity to DDA in ovarian cancer (OC)[6] and EWS[11, 12] as well as colorectal cancer[13, 14] and lung cancer.[15]

At the same time, SLFN11 expression was being correlated with response to PARP inhibitors in lung cancer cells[15, 16]. Recently, this latest finding was also confirmed in a phase II double-blind randomized study that showed an improved progression free-survival (5.7 months vs 3.6 months) and overall survival (12.2 months vs 7.5months) in the SLFN11-positive small cell lung cancer patients compared with the SLFN11-negative tumors.[5]

Taken together, all the published data point toward the role of SLFN11 as a bona fide predictive biomarker of response to DDA including platinum salts, topoisomerase inhibitors, alkylating agents and PARP inhibitors. However, in spite of the extensive literature accumulated thus far, no reliable immunohistochemistry (IHC) protocols to evaluate SLFN11 expression in formalin-fixed paraffin-embedded (FFPE) material have been established, with the ensuing risk of declaring the medical utility of SLFN11 staining in cancer without a previous phase of rigorous analytical validation.

2) Assessment of SFLN11

To date, several studies had evaluated gene expression, mRNA expression, and protein expression of SFLN11 in different cancer cell lines and tumor samples showing a good correlation between

SLFN11 transcript expression, measured by quantitative real time polymerase chain reaction (qRT-PCR), and SLFN11 protein levels, evaluated by Western Blot (WB) or IHC. (3,5,6)

In none of these works a systematic, analytically designed procedure for FFPE staining of SLFN11 by IHC was reported.

Actually, widely different antibody sources, clones, product codes and concentrations were utilized in IHC and WB experiments to assess SLFN11 expression in cancer cell lines and tumor samples as showed in the Table N.1. Furthermore, the descriptions of SLFN11 assessment in supplementary data of different studies are often not exhaustive, and in at least two occasions, different antibodies were employed in the same paper.[17]

Finally, different IHC scores[5,15,16,18] have been arbitrarily applied, but a standardized score for SLFN11 evaluation in human tissues has neither been validated nor approved by a consensus of researchers.

Interestingly, IHC scores were created in different manners.[14,17,16] Even, in a recent publication the Authors applied an ad hoc modified IHC score[5,16] (see Table N.1) that presented an improved correlation of SLFN11 with clinical data.

All the data published concerning the heterogeneity of SLFN11 assessment with the commercially available antibodies, their dilutions and proposed uses have been summarized in Table N.1.

Unfortunately, the absence of unanimously validated antibodies, of a reliable IHC staining protocol and the subsequent differences in construction and use of staining scores converge in a lack of uniformity in SLFN11 evaluation. Due to the increasingly evident relevance of SLFN11 as a potential predictive biomarker for DDA activity, there is an urgent need to standardize its assessment in order to avoid a new “Ki-67 situation”. The proliferation marker Ki-67, in different studies[19-22], is universally used as a prognostic, clinically useful prognostic biomarker in breast cancer. Ki-67 however has been the subject of never ending debates due to considerable

interlaboratory assessment differences, the use of different antibodies over the years for IHC staining, an inter and intra-observer variation in the definition of Ki-67 score (some pathologists estimate the percentage of nuclei staining; others count several hundred nuclei in different areas of tumours to give an overall average index; elsewhere automated readers are used). Finally, an optimal cut-off for defining Ki-67 as "high" has lacked for several years, and even now the debate has not completely subsided.[23,24]

To avoid a delay in the clinical validation of SLFN 11 as predictive biomarker, we advocate that the following steps be rigorously performed in sequence: 1) Analytical (technical) validation of an IHC staining method; 2) Clinical (biological) validation in selected retrospective-prospective cohorts such as biobank from large, well-conducted phase III trials and 3) Clinical utility demonstration through prospective companion studies in clinical trials. The two latest steps can only have value if following international recommendations[20,25], and after the generation of a reliable, agreed upon IHC staining protocol as well as an optimal IHC scoring system.[26]

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Table

Provider and product name	***Source***	***Studies***	***WB, Dl***	***IHC, Dl***	***IHC score***	***IMF***
NBP1-92368 NB	Rabbit Poly	2) Li M et al. 2012*	2) Yes, N.S	2) No		2) No
HPA-023030 SA	Rabbit Poly	6) Barretina J et al. 2012	6) Yes, 1:500	6) No	6) No	7) No
		7) Lok BH et al. 2017#	7) No	7) Yes, n.s.	7) Yes[I]	8) No
		8) Gardner EE et al. 2017	8) No	8) Yes, N.S	8) Yes[II]	9) No
		9) Stewart CJR el al. 2017	9) No	9) Yes, 1:50	9) Yes[III]	10) No
		10) Pietanza MC et al. 2018	10) No	10) Yes N.S	10) Yes[IV]	11) No

SC-136891 (K-13)	Goat Poly	3) Zoppoli G et al. 2012 4) Tian et al. 2014	3) Yes, 1:500 4) Yes, N.S	3) No 4) No		3) Yes 4) No
SC-374339 (E-4)	Mouse Mono	12) Abdel-Mohsen M et al. 2013 13) Tang SW et al. 2015 14) Kang MH et al. 2015 15) Goss Kl et al. 2016 16) Murai J et al. 2016 17) Nogales V et al. 2016 18) He T et al. 2017 19) Lok BH et al. 2017 20) Gardner EE et al. 2017 21) Tang SW et al. 2018& 22) Valdez F et al. 2018*	12) Yes, 1:500 13) Yes, N.S. 14) Yes, N.S 15) Yes, 1:500 16) Yes, N.S 17) Yes, N.S 18) Yes, 1:200 19) Yes, 1:250 20) Yes, 1:250 21) Yes 22) Yes, 1:500	12) No 13) No 14) No 15) No 16) No 17) No 18) No 19) No 20) No 21) No 22) No		12) No 13) No 14) No 15) No 16) No 17) Yes 18) No 19) No 20) No 21) No 22) Yes, 1:200
Ab-121731	Rabbit Poly	2) Deng Y et al. 2017	2) No	2) Yes	Yes $	6) No
SC- 515071 (D-2)	Mouse Mono	4) Tang SW et al. 2018 5) Murai J et al. 2018** 6) Valdez F et al. 2018	4) Yes& 5) Yes, 1:10000 6) Yes,	2) No		4) No 5) Yes,1 :1000 6) Yes, 1:200

			1:500			
SC-136890	Goat Poly	1) Ferraioli et al. 2018	1) No	1) Yes, 1:100	1) YesΣ	1) No

Table N. 1: Anti-Schlafen 11Antibodies

Ab: Abcam, Dl: dilution, IHC: immunohistochemistry, I: Intensity, IMF: Immunofluorescence, Mono: Monoclonal, NB: Novus biological, N.S: not specified, P: Positive, Poly: Polyclonal, SA: Sigma-Aldrich, SC: Santa Cruz, WB: Western Blot.

***:** Studies in infectious disease (HIV-virus)
****:** Immunoprecipitation (IP) and IP coupled to Mass Spectrometry: Anti-Schlafen11 antibody SC- 374339 (E-4)
#: Modified H-score, Dichotomized H-Score
$: Intensity was scored as 0, none; 1, weak; 2: moderate; 3, strong. The proportion of positive tumor cells was assigned to 0 (<25 % positive), 1 (25–50 % positive), 2 (50–75 % positive), 3 (75–100 % positive). The final score was calculated by intensity plus proportion (0–6).
&: In this paper the authors don't clearly describe how and when they used two different antibodies
I: Three intensities of IHC nuclear staining and their frequency. A final expression (H-score) from 0 to 300 ,
II: The same score of Lok et al. (2017): Three intensities of IHC nuclear staining and their frequency. A final expression (H-score) from 0 to 300
III: Nuclear expression of SLFN11 were quantified using a 4-value intensity score (0, none; 1, weak; 2, moderate; and 3, strong) and the percentage (0%–100%) of the extent of reactivity. A final expression score (H-score) was obtained by multiplying the intensity and reactivity extension values (range, 0–300)
IV: Sections were scored for intensity (0-3+) and extent (0- 100%) of staining by light microscopy. By multiplying intensity and extent of staining, each tumor was assigned an H-score (range 0-300). For SLFN11, an immunohistochemistry (IHC) score of 1 or greater was considered positive.
Σ: Intensity Score (IS):0: no stain,1+: weak stain (visible at high magnification), 2+: moderate stain (visible at scan magnification), 3+: intense stain (Tumor Infiltrating Lymphocytes –TIL); Distribution Score (DS):0: no stained cells,1+: <10% of stained cells,2+: 10-40% of stained cells,3+: >40% of stained cells. Histological Score (HS)was obtained: HS=IS x DS. HS=0 SFLN11 Negative, 0<HS≤2 SFLN11 Low, 2<HS<6 SFLN11 Intermediate, HS≥6 SFLN11 High

7) Conclusion

- SLFN11 is a new putative helicase DNA/RNA protein discovered by Zoppoli and Barretina in the year 2012.
- SLFN11 expression is causally associated with response to DDA in cancer cells.
- SLFN11 is induced by IFN, but the current relationship between TILs and SLFN11 expression in cancer tissues is not known.
- Hypermethylation of CpG promoter island, that are located around its transcription start site, inhibits SLFN11 expression.
- In IHC and WB, several antibodies and different scores have been used to evaluate SLFN 11 protein expression but nobody has been clearly validated.
- A linear relationship between mRNA measured by qRT-PCR and protein expression (WB and IHC) derived by activation of SLFN 11 gene has been confirmed.
- Several preclinical and clinical models point toward SLFN11 as a predictive marker of response to DDA and PARP inhibitors.
- At present, the predictive role of SLFN11 expression in human tumors is unclear and needs further investigation but it seems that SLFN11 induces lethal replication block in response to a broad type of DNA-targeting agents and PARP inhibitors.

Further studies will be performed to confirm our hypothesis in order to: 1) better understand the function of SLFN 11 in cancer cell, 2) validate an easy, reliable and standardized IHC protocol to assess SLFN11 expression, 3) use SFLN11 expression as a predictive biomarker of response to DDA and PARP inhibitors and 4) determinate the relationship with immune system.